REPORT DOCUMENTATION PAGE

AFRL-SR-AR-TR-03-

0288

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
July 1, 2003	Final Technical	8/15/02-12/31/02

4. TITLE AND SUBTITLE
10th International Conference on Retinal Pigments

5a. CONTRACT NUMBER

5b. GRANT NUMBER
F49620-02-1-0392

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
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5d. PROJECT NUMBER
2313

5e. TASK NUMBER
AX

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Washington
Seattle, WA 98195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFOSR
4015 Wilson Blvd, Room 713
Arlington VA 22203-1954

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approve for Public Release: Distribution Unlimited

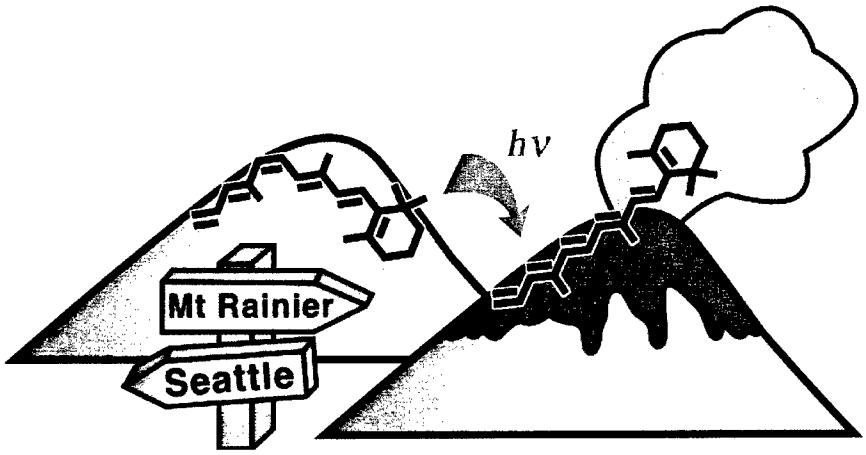
13. SUPPLEMENTARY NOTES
upasaka2543

14. ABSTRACT
The meeting was a resounding success with the largest attendance ever, over 200 Scientists, representing 12 countries. Three copies of the conference program, abstract book and list of participants are enclosed.

20030822 139

15. SUBJECT TERMS

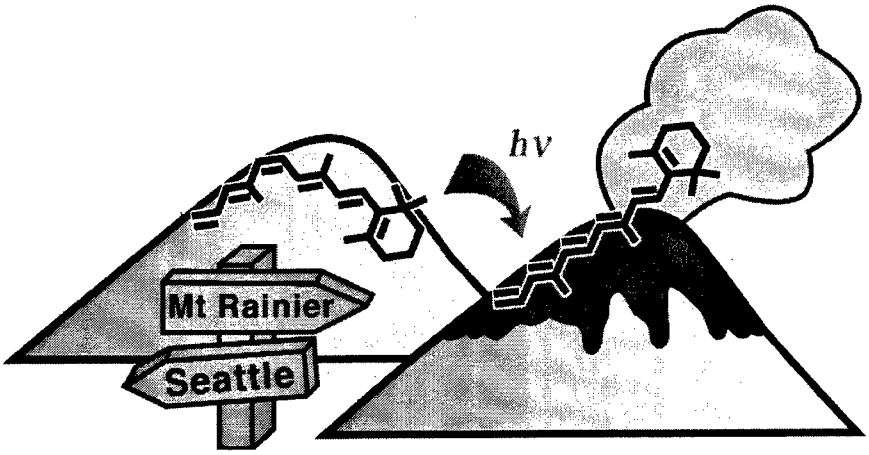
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Thomas G. Ebrey
					19b. TELEPHONE NUMBER (include area code) 206-685-3550



10th INTERNATIONAL CONFERENCE ON RETINAL PROTEINS

PROGRAM AND ABSTRACTS

University of Washington
Seattle, USA
August 20-24, 2002



10th INTERNATIONAL CONFERENCE ON RETINAL PROTEINS

PROGRAM AND ABSTRACTS

University of Washington
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INTERNATIONAL ADVISORY COMMITTEE

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Dear Participants,

On behalf of the Organizing Committee, I want to welcome you to the 10th International Conference on Retinal Proteins, held on the Campus of the University of Washington.

A few words on the origin of this Conference, which brings together people studying different Retinal Proteins in all their glorious manifestations. The first time the rhodopsin people admitted others to their select society was probably the 1st US-Japan meeting on Vision that Toru Yoshizawa and I organized at Urbana (Illinois) in 1975. We had heard of this "bacteriorhodopsin" and invited Walther Stoeckenius to tell us all about it. His missionary work fell on fertile ground and several rhodopsin people started working on bacteriorhodopsin. There was also some mixing of the pigment people at a meeting in Ischia, Italy soon thereafter. But surely the first full blown meeting which started the series of the current Retinal Protein Conferences was the one organized by Dieter Oesterhelt near Munich in 1984. This was so successful that a second meeting was held at Lake Baikal, Russia in 1986 and a third at Mt. Hiei, Kyoto two years after that. At that meeting it was decided to make this a regular every two year event and the next one was set for Santa Cruz, California (1990), then Dourdan, France (1992), Amsterdam (1994), Israel (1996), Awaji Island, Japan (1998), and Szeged, Hungary (2000).

Each of these meetings heralded important advances to the Retinal Protein field and stimulated the development of the new ones. I hope that our meeting will keep up this tradition. These are indeed very exciting times for all Retinal Protein people. New pigments are being discovered, new structures are appearing, new methods are being employed, new data are giving important insights, and the new theoretical work is becoming more and more illuminating. Enjoy.

Tom Ebrey,

Chair of the Organizing Committee

PROGRAM

ARRIVAL DAY, TUESDAY, AUGUST 20

Registration at Terry-Lander Hall	1:00 pm - 5:30 pm
Reception/Buffet Dinner at the Faculty Club	6:00 pm - 8:00 pm

FIRST DAY, WEDNESDAY, AUGUST 21

7:00 - 8:30	BREAKFAST
9:00 - 9:10	**Meeting opening**
9:10 - 12:15	Session I: **Regulation of the Chromophore's Absorption Spectrum in Retinal Proteins** Chair - ***Daniel Oprian/David Hunt***
9:10 - 9:20	***Daniel Oprian***. Introduction.
9:20 - 9:45	**L-1: *Shozo Yokoyama***. Molecular genetics and evolution of ultraviolet vision in vertebrates.
9:45 - 10:10	**L-2: *Jeffry Fasick***. Spectral tuning in the mammalian short-wavelength sensitive cone pigments.
10:10 - 10:35	**L-3: *Robert Birge***. Wavelength regulation in the blue & UV cone pigments and sensory rhodopsin II.
10:35 - 11:00	COFFEE BREAK
11:00 - 11:25	**L-4: *Phyllis Robinson***. Molecular characterization of opsins from aquatic mammals: Insights into spectral tuning mechanisms and the evolution of mammalian color vision.
11:25 - 11:50	**L-5: *Mordechai Sheves***. Factors controlling color regulation and light-induced dipole in retinal proteins.
11:50 - 12:15	**L-6: *Klaus Schulten***. Spectral tuning and photodynamics in visual receptors.
12:15 - 2:20	LUNCH AND POSTER MOUNTING
2:20 - 5:30	Session II: **Photocycle and Proton Movements in Bacterio-rhodopsin** Chairs - ***Pál Ormos/Hemi Gutman***
2:20 - 2:30	***Pál Ormos***. Introduction.

2:30 - 2:55 **L-7: *László Zimányi***. The bacteriorhodopsin photocycle revisited.

2:55 - 3:20 **L-8: *Judith Herzfeld***. Solid state NMR studies of the proton transport mechanism in bacteriorhodopsin.

3:20 - 3:45 COFEE BREAK

3:45 - 4:10 **L-9: *György Váró***. Comparing different photocycle models.

4:10 - 4:35 **L-10: *Sergei Balashov***. Changing the pK_a's in the photocycle of bacteriorhodopsin (BR); participation of Thr205 and Ser193 in light-driven proton transport.

4:35 - 4:50 **L-11: *Esther Nachliel***. The effect of surface mutation on 'proton hole' propagation dynamics in photo activated bacteriorhodopsin.

Creation and Destruction of Bacteriorhodopsin

4:50 - 5:15 **L-12: *Mark Krebs***. Cellular and structural determinants of bacteriorhodopsin biogenesis.

5:15 - 5:30 **L-13: *Norbert Dencher***. Bacteriorhodopsin and oxidative stress: examining the membrane theory of aging.

5:30 - 7:30 DINNER

7:30 - 8:55 Session III: **Primary Photochemical Events in Retinal Pigments**
Chair - ***Johan Lugtenburg***

7:30 - 7:40 ***Johan Lugtenburg***. Introduction.

7:40 - 8:05 **L-14: *Toshiaki Kakitani***. Twist-sharing one-bond rotation (TSOR) mechanism in the *cis-trans* photoisomerization of rhodopsin.

7:05 - 8:30 **L-15: *Sandy Ruhman***. Unraveling the primary events in photoexcited bacteriorhodopsin using stimulated emission pumping.

8:30 - 8:55 **L-16: *Richard Mathies***. Molecular mechanism of rhodopsin isomerization and protein activation from resonance Raman spectroscopy.

9:00 - 11:00 KOJI NAKANISHI'S MAGIC TRICKS. BEER/WINE/SOFT DRINKS BY POSTERS.

SECOND DAY, THURSDAY, AUGUST 22

7:00 - 8:30	BREAKFAST
9:00 - 11:40	Session IV: **Origin, Evolution and Diversity of Visual Pigments** Chair - **Tom Ebrey**
9:00 - 9:10	***Tom Ebrey***. Introduction.
9:10 - 9:35	**L-17: *Thomas Sakmar***. Recreating functional ancestral archosaur visual pigments.
9:35 - 10:00	**L-18: *Motoyuki Tsuda***. Three distinct retinal proteins expressed in the brain of the primitive chordate, ascidian.
10:00 - 10:25	**L-19: *Akihisa Terakita***. Comparative study on the diversity in rhodopsin family.
10:25 - 10:50	COFFEE BREAK
10:50 - 11:15.	**L-20: *Osamu Hisatomi***. Diversity and evolution of vertebrate phototransduction systems.
11:15 - 11:40	**L-21: *Yoshinori Shichida***. The difference in molecular properties between rod and cone visual pigments.
11.40 - 12.00	PICTURE TAKING
12:00 - 2:00	LUNCH AND POSTERS
2:00 - 5:30	Session V: **Structure of Halobacterial Retinal Proteins and their Photointermediates** Chair - ***Tsutomu Kouyama/Robert Glaeser***
2:00 - 2:10	***Tsutomu Kouyama***. Introduction.
2:10 - 2:35	**L-22: *Joerg Tittor***. Why does nature form a purple membrane?
2:35 - 3:00	**L-23: *Ehud Landau***. Structural dynamics of retinal proteins: lessons from X-ray crystallography and microspectrophotometry.
3:00 - 3:25	**L-24: *Sriram Subramaniam***. Electrons vs. X-rays in the analysis of protein conformational changes in bacteriorhodopsin.
3:25 - 3:50	COFFEE BREAK

3:50 - 4:15	**L-25:** ***Mikio Kataoka***. Structure and properties of photo-intermediate and relationship to the photoreaction.
4:15 - 4:40	**L-26:** ***Georg Bueldt***. High resolution structures of intermediate states of bacteriorhodopsin.
4:40 - 5:05	**L-27:** ***Janos Lanyi***. Crystallographic description of the photointermediates of bacteriorhodopsin.
5:05 - 5:30	**L-28:** ***Hudel Luecke***. Light-driven ion pumping and signaling in bacterial rhodopsins.
5:30 - 7:30	DINNER AT THE FACULTY CLUB
7:30 - 8:55	Session VI: **Photoactive Yellow Protein (PYP)** Chair - ***Roberto Bogomolni***
7:30 - 7:40	***Roberto Bogomolni***. Introduction.
7:40 - 8:05	**L-29:** ***Klaas Hellingwerf***. The mechanism of photoactivation of yellow proteins.
8:05 - 8:30	**L-30:** ***Jun Sasaki***. What triggers the protein folding in PYP_M-to-PYP_{dark} conversion: Investigations using Met100 mutants.
8:30 - 8:55	**L-31:** ***Aihua Xie***. Electrostatic interactions in photoreceptor activation.
9:00 - 10:30	BEER/WINE/SOFT DRINKS BY POSTERS

THIRD DAY, FRIDAY, AUGUST 23

7:00 - 8:30	BREAKFAST
9:00 - 10:50	Session VII: **Rhodopsin Structure** Chair – ***Ron Stenkamp/Ehud Landau***
9:00 - 9:10	***Ron Stenkamp***. Introduction.
9:10 - 9:35	**L-32: *David Teller***. Three dimensional structure of rhodopsin.
9:35 - 10:00	**L-33: *Gebhard Schertler***. Structure of native bovine rhodopsin in a P3(1) crystal form.
10:00 - 10:25	**L-34: *Philip Yeagle***. Three dimensional structure and activation of rhodopsin.
10:25 - 10:50	**L-35: *Willem DeGrip***. Studies on structure and mechanism of rhodopsin by FT-IR and solid state NMR spectroscopy.
10:50 - 11:15	COFFEE BREAK
11:15 - 3:35	Session VIII: **Retinal Processing for Visual Pigment Regeneration** Chair - ***Jack Saari***
11:15 - 11:25	***Jack Saari***. Introduction.
11:25 - 11:50	**L-36: *Kris Palczewski/Yoshikazu Imanishi***. Flow of retinoids in the vertebrate retina.
11:50 - 12:15	**L-37: *Carter Cornwall***. Vitamin A, the visual cycle, and dark adaptation.
12:15 - 2:00	LUNCH AND POSTERS
2:00 - 2:25	**L-38: *Henry Fong***. Interactions of the RGR opsin with a retinal chromophore.
2:25 - 2:50	**L-39: *Jian-xing Ma***. A novel all-*trans* retinol dehydrogenase in the photic visual cycle of retinoid metabolism.
2:50 - 3:15	**L-40: *Koji Nakanishi***. Rhodopsin and age-related macular degeneration pigments.

3:15 - 3:40	**L-41: *Rosalie Crouch***. Chromophore interactions with rod and cone opsins.
3:40- 4:05	COFFEE BREAK
4:05 - 5:30	Session IX: **Vibrational Spectroscopy of Retinal Proteins I** Chair - ***Akio Maeda***
4:05 - 4:15	***Akio Maeda***. Introduction.
4:15 - 4:40	**L-42: *Hideki Kandori***. Internal water molecules of rhodopsins in action.
4:40 - 5:05	**L-43: *Klaus Gerwert***. Proton transfer in bacteriorhodopsin via protonated H-bonded networks of internal water molecules.
5:05 - 5:30	**L-44: *Friedrich Siebert***. Activation and photocycling of 11-*cis*-locked rhodopsin.
5:30 - 7:30	DINNER
7:30 - 8:55	Session X: **Vibrational Spectroscopy of Retinal Proteins II** Chair - ***Andrei K. Dioumaev***
7:30 - 7:40	***Andrei K. Dioumaev***. Introduction.
7:40 - 8:05	**L-45: *Esteve Padrós***. Structure-function relationships in the extracellular domain of bacteriorhodopsin.
8:05 - 8:30	**L-46: *Mark Braiman***. Proteorhodopsin and bacteriorhodopsin: similarities and differences in structure and function.
8:30 - 8:55	**L-47: *Joachin Heberle***. Time-resolved vibrational analysis of the photoreaction of proteorhodopsin from marine bacterioplankton.
9:00 - 10:30	BEER/WINE/SOFT DRINKS BY POSTERS. REMOVAL OF THE POSTERS.

FOURTH DAY, SATURDAY, AUGUST 24

7:00 - 8:30	BREAKFAST
9:00 - 11:40	Session XI: **Microbial Rhodopsins: Diversity and Mechanisms** Chairs - ***John Spudich/Naoki Kamo***
9:00 - 9:10	***John Spudich***. Introduction.
9:10 - 9:35	**L-48: *John Spudich***. Microbial rhodopsins: genome-mining, diversity, and structure/function relationships.
9:35 - 10:00	**L-49: *Martin Engelhard***. Structural insight into the early steps of receptor-transducer signal transfer.
10.00 - 10:25	**L-50: *Leonid Brown***. Photochemical transformations of *Neurospora* rhodopsin, an eucaryotic homologue of halobacterial photosensors.
10:25 - 10:50	COFFEE BREAK
10:50 - 11:15	**L-51: *Oleg Sineshchekov***. Two rhodopsins mediate motility responses at low and high intensity light in green flagellated algae.
11:15 - 11:40	**L-52: *Ernst Bamberg***. Proteorhodopsin and channelopsin I: vectoriality and light activated channel function.
11:40 - 12:40	LUNCH
12:40 - 4:35	Session XII: **Activation by Rhodopsin** Chair - ***Jim Hurley/Nadik Abdulaev***
12:40 - 12:50	***Nadik Abdulaev***. Introduction.
12:50 - 1:15	**L-53: *David Kliger***. The mechanism of rhodopsin activation as revealed by time-resolved absorption studies.
1:15 - 1:40	**L-54: *Jack Sullivan***. Rhodopsin activation as seen through the eye of a microelectrode.
1:40 - 2:05	**L-55: *Klaus Peter Hofmann***. Metabolic versus light-dependent deactivation of vertebrate rhodopsin.
2:05 - 2:30	**L-56: *Paul Liebman***. Biochemistry underlying uniformity of rod single photon responses.

2:30 - 2:55	**L-57: *Kevin Ridge***. Mapping interactions between carboxyl-terminal peptides of the alpha-subunit of transducin and a functional mimic of light-activated rhodopsin.
2:55 - 3:20	COFFEE BREAK
3:20 - 3:45	**L-58: *Ulrike Alexiev***. Conformational changes in rhodopsins: An investigation with time-resolved fluorescence depolarization and absorption spectroscopy.
3:45 - 4:10	**L-59: *David Farrens***. Insights into the role of the "retinal plug".
4:10 - 4:35	**L-60: *Gobind Khorana***. A structural basis for a common mechanism of activation in G-protein coupled receptors.
4:35 - 5:00	**Closing of the conference**
5:30 - 10:00	EXCURSION AND BANQUET (boat trip to Tillicum Village on Blake Island, harbor tour, wine reception, traditional Indian style salmon dinner and Native American Dance show).

SUNDAY, AUGUST 25

DEPARTURE

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ABSTRACTS OF LECTURES

L-1 MOLECULAR GENETICS AND EVOLUTION OF ULTRAVIOLET VISION IN VERTEBRATES

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Vertebrates use UV vision for such basic behaviors as mating, foraging, and predation. Despite its biological importance, the molecular basis of UV vision was unknown until recently. We have transformed the UV pigment of the zebra finch (*Taeniopygia guttata*) into a violet pigment by incorporating only one amino acid change, C90S. By incorporating the reverse mutations, we have also constructed UV pigments from the orthologous violet (SWS1) pigments of the pigeon (*Columba livia*) and chicken (*Gallus gallus*), showing that the spectral tuning in avian UV pigments is controlled by one amino acid site. More recently, we successfully interchanged the color-sensitivities of the mouse UV pigment and the human blue pigment by introducing forward and reverse mutations at amino acid sites 46, 49, 52, 86, 93, 114, and 118. So far, we have identified a total of 10 amino acid sites that are involved in the spectral tuning in the SWS1 pigments.

Comparative sequence analyses and actual reconstruction of the ancestral SWS1 pigments show that most contemporary UV pigments inherited their UV sensitivities from the vertebrate ancestor by retaining most of these 10 amino acids. In the avian lineage, the ancestral pigment lost UV sensitivity, but some descendants regained it by S90C.

L-2 SPECTRAL TUNING IN THE MAMMALIAN SHORT WAVELENGTH SENSITIVE CONE PIGMENTS

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The wild-type mouse ultraviolet (UV) and bovine blue cone visual pigments have absorption maxima of 358 and 438 nm, respectively, while sharing 87% amino acid identity. To determine the molecular basis underlying the 80 nm spectral shift between these pigments, we selected several amino acids in helices II and III for site-directed mutagenesis. These amino acids included those that differ between mouse UV and bovine blue, as well as the conserved counterion, Glu113. These studies resulted in the identification of a single amino acid substitution at position 86 responsible for the majority of the spectral shift between the mouse UV and bovine blue cone pigments. This is the first time that this amino acid by itself has been shown to play a major role in the spectral tuning of the SWS1 cone pigments. A single amino acid substitution appears to be the dominant factor by which the majority of mammalian short-wavelength sensitive cone pigments have shifted their absorption maxima from the UV to the visible regions of the spectrum. Studies investigating the role of the conserved counterion Glu113 suggest that the bovine and mouse SWS1 pigments result from a protonated and unprotonated Schiff base chromophore, respectively.

L-3 WAVELENGTH REGULATION IN THE BLUE & UV CONE PIGMENTS AND SENSORY RHODOPSIN II

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Cone pigments in the SWS1 group mediate visual sensitivity in the short wavelength region of the spectrum, exhibiting absorption maxima between 350-450 nm. The SWS1 pigments are the only opsins with absorption maxima blue-shifted compared to a protonated retinal Schiff base in aqueous solution, (~440 nm). Mouse UV (MUV) has an absorption maximum at ~357 nm, and we demonstrate that it has an unprotonated Schiff base linkage. In contrast, the *Xenopus* violet cone (VCOP) is shown to have a protonated Schiff base linkage. MUV and VCOP share significant homology and thus small changes in the local chromophore environment can be studied in detail. We use a combination of site directed mutagenesis and quantum theory to reveal the principal mechanisms of wavelength selection in these two pigments. We also examine wavelength regulation in sensory rhodopsin II (SRII). SRII is unique among the archaeal rhodopsins in having an absorption maximum near 500 nm, roughly 70 nm blue shifted from the other pigments. We conclude that the major source of the blue shift is associated with significantly different positions of Arg-72 (Arg-82 in bR) in the two proteins, but that protein-chromophore interactions involving Trp-76, Tyr-174, Asp-201, Thr-204 and W-402 also contribute to the blue shift.

L-4 MOLECULAR CHARACTERIZATION OF OPSINS FROM AQUATIC MAMMALS: INSIGHTS INTO SPECTRAL TUNING MECHANISMS AND THE EVOLUTION OF MAMMALIAN COLOR VISION

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It has long been hypothesized that the visual systems of animals are evolutionarily adapted to their visual environment. The entrance many millions of years ago of certain mammals into the sea gave these new marine mammals completely novel visual surroundings with respect to light availability and predominant wavelengths. Work from our laboratory has examined the evolutionary changes in visual pigments that occurred as two orders and one suborder of mammals with different terrestrial ancestors moved from a land to an aquatic environment. This comparative approach has revealed interesting results regarding the design of both the photopic and scotopic visual systems in *Cetacea, Sirenia* and *Pinnipedia*. Whales and seals lack dichromatic color vision due to the expression of only a single cone opsin gene. This is an unexpected finding for color vision appears to be under strong selective pressure in diurnal terrestrial mammals. In contrast, the manatee, a representative from the order *Sirenia,* has an intact SWS gene, which is expressed in the retina. In addition, by elucidating the mechanisms of wavelength modulation in both the rod and cone opsins from these aquatic mammals, we discovered a novel tuning mechanism in *Cetacean* long-wavelength sensitive (LWS) visual pigments. Our work on the molecular basis for changes in the spectral sensitivity of rod visual pigments show a relationship between blue-shifted rhodopsins, deep-diving foraging and substitutions at three key tuning sites.

L-5 FACTORS CONTROLLING COLOR REGULATION AND LIGHT- INDUCED DIPOLE IN RETINAL PROTEINS

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The absorption maxima of retinal proteins are spread over wide range due to specific chromophore protein interactions. Bacteriorhodopsin(bR) for example absorbs at 568nm, and it was suggested that the red shift relative to absorption of the chromophore in solution (440nm in methanol) is due to retinal ring-chain s-trans planarization and weak protonated Schiff base-counterion interaction. In contrast, Sensory Rhodopsin (NpSR2) absorbs at 500nm.The high resolution crystal structures suggest that both bR and NpSR2 share similar ring-chain conformation and very small deviation in Schiff base-counterion distance.

We have performed model studies in solution which suggest that the absorption maximum of retinal protonated Schiff base is influenced by the specific angle between the protonated Schiff base linkage and its counterion thereby controlling the hydrogen bonding efficiency introduced by the bridging water molecule.

The significant red shifts detected in retinal proteins is based on a large light induced dipole developed in the retinal chromophore following light absorption. Second harmonic generation measurements of several artificial pigments indicate that the large light induced dipole is due to chromophore protein interactions in the vicinity of the retinal ring. We suggest that Tryptophan residues located in the ring vicinity are responsible for the enhanced excited state charge delocalization and large light induced dipole.

L-6 SPECTRAL TUNING AND PHOTODYNAMICS IN VISUAL RECEPTORS

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Detection of light is essential for many organisms. The recently obtained high resolution structures of rhodopsin (Rh) make it possible to describe at the electronic and atomic levels the functional processes in a visual receptor stretching over many time scales. The main objective of research on Rh is now to identify the physical mechanism of **spectral tuning** in color vision and phototaxis, to reveal in unprecedented detail the ultrafast **photoisomerization dynamics** of the chromophore retinal that triggers the functional processes, and to elucidate the **Rh activation process** that links light absorption to transducin binding.

Recently, we have achieved progress in regard to those issues using computational methods such as high-level *ab initio* quantum mechanical/molecular mechanical (QM/MM) excitation and molecular dynamics (MD) calculations as well as large-scale long-time MD simulations. In regard to **spectral tuning** we have identified structural determinants of the observed large difference in absorption maxima of bacteriorhodopsin (bR) and sensory rhodopsin II [1], building on the available crystal structures. In regard to **photoisomerization dynamics** we have recently achieved the first ever *in situ* simulation of the photoisomerization dynamics of retinal, actually an analogue, in bR by means of an *ab initio* QM/MM MD method. This calculation unveiled in complete molecular detail the anomalously fast event, including the molecular origin of dynamic spectral modulations observed in novel ultrafast spectroscopic experiments. In regard to the **Rh activation process** we have modeled the early intermediate states of bR's photocycle, K and KL, revealing the molecular mechanism of the photoenergy storage and key conformational changes that facilitate later proton transfers [2]. We have also investigated through 10 ns MD simulations of a fully hydrated model of membrane-embedded Rh the response of the protein to retinal's photoisomerization [3].

[1] Hayashi et al. *J. Phys. Chem. B* 105, 10124 (2001). [2] Hayashi et al. *Biophys J.* 83 (2002). In press. [3] Saam et al. *Biophys J.* 83 (2002). In press.

L-7 THE BACTERIORHODOPSIN PHOTOCYCLE REVISITED

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The photocycle of wild type bacteriorhodopsin is still a puzzle waiting to be accurately solved, despite many past attempts, which provided a large body of controversial results. We have pursued this task by analyzing kinetic multichannel difference spectra in the visible spectral range, following a strategy to obtain the intermediates' absorption spectra first, after which the determination of their time evolution is all but trivial. Finally, the intermediates' kinetics are fitted by various reaction schemes (a.k.a. photocycle models). Due to the complexity of the photocycle and the rather structureless and overlapping intermediate spectra, direct global fits to the raw data by assuming photocycle models has only been fully successful when analyzing simulated data. Our approach, which tries to determine the intermediate spectra without the assumption of a photocycle model, has recently been applied to the D96N and E204Q mutant proteins.

The method of singular value decomposition with exponential fit assisted self-modeling (SVD-EFASM) will be introduced and applied to data sets obtained on wild type BR. New data were measured on oriented purple membrane sheets with appropriate measuring geometry and actinic laser po larization. This arrangement minimizes the complication of heterogeneous reaction kinetics expected in random samples due to orientation-dependent excitation. SVD-EFASM reveals the stoichiometric behavior of the intermediates, and yields so far hidden minor spectral differences between intermediate substates. These spectral details may be the key to obtain a satisfactory description of the photocycle, which also has to take into account all accumulated knowledge about the steps of the proton pumping mechanism.

L-8 SOLID STATE NMR STUDIES OF THE PROTON TRANSPORT MECHANISM IN BACTERIORHODOPSIN

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The successive M_O and M_N states of bacteriorhodopsin obtained by illumination of native purple membranes at low temperature, show spectral characteristics indicating that they bracket the switch in the connectivity of the Schiff base between the two sides of the membrane — i.e., that they are functionally early and late M states respectively. It is therefore expected that comparisons between the M_O and M_N states will afford insights into the enforcement of vectorial action in the ion pump cycle. Our work focuses on features of the M_O and M_N states that can be probed by a variety of solid state NMR experiments. These include interactions reflected in chemical shifts, inter-nuclear distances derived from dipolar couplings, and torsion angles based on the evolution of double quantum coherence under the influence of dipolar interactions. From its ^{15}N chemical shift, we infer that the Schiff base is interacting with a hydrogen bond donor in both the M_O and M_N states. This interaction is stronger in the M_N state, consistent with imminent reprotonation. However, the presence of a hydrogen bond donor in the M_O state suggests association with water and the possibility of inward transport of OH^- rather than outward transport of H^+. Torsion angle measurements show that both the C_{14}–C_{15} and the C_{15}=N bonds of the chromophore are distorted from planar in the light-adapted state. The C_{14}–C_{15} bond becomes still more distorted in the M_O state, but it does not change significantly in the $M_O \rightarrow M_N$ transition. This is consistent with evidence that it is torsion in the nominal double bonds of the chromophore that relaxes in the $M_O \rightarrow M_N$ transition. Other changes in the $M_O \rightarrow M_N$ transition include the environment of the C_{20} methyl group of the retinal and the hydrogen bonding of at least two of the indole nitrogens. However, there is essentially no change in the hydrogen bonding of the indole nitrogen of W182 even though it abuts C_{20}.

L-9 COMPARING DIFFERENT PHOTOCYCLE MODELS

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The photocycle of different retinal proteins, such as the proton transporting bacteriorhodopsin and proteorhodopsin, or the chloride ion transporting salinarum and pharaonis halorhodopsin are compared. The changes are followed, when the external conditions are altered. It is shown that the different environmental conditions or the different mutants alter the ion transport capability of the protein. The transient difference spectra, the absorption kinetic signals and the light induced electric response signals were measured. From these the spectra of intermediates, a photocycle model and the electrogenicity of each intermediate was calculated, which describes the steps of the ion transport across the cell membrane. From the accumulated data general information was concluded about the requirements to have a non-transporting, or chloride ion transporting, or proton pumping photocycle. The fast, red shifted, K-like intermediate appears in all the studied photocycles and it has negative electrogenicity. The ion transport is indicated by the existence of the slow intermediate (N, O), having a large positive electrogenicity. Proton transport is indicated by the existence of intermediate M, characteristic to the deprotonated Schiff-base.

L-10 CHANGING THE pK_a's IN THE PHOTOCYCLE OF BACTERIORHODOPSIN (BR); PARTICIPATION OF THR205 AND SER193 IN LIGHT-DRIVEN PROTON TRANSPORT

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Proton transport in bR is initiated by light-induced chromophore isomerization and is driven by changes in the accessibility and proton affinities of the key groups: the Schiff base, Asp85, Asp96 and proton release complex (PRC). The latter includes Arg82, Glu204, Glu194 and water molecules. The light-induced H^+ gradient produced by bR depends on the difference in the pK_a's of the groups which release and take up a H^+. Study of the pH dependencies of H^+ uptake and photocycle reactions enabled us [1,2] to determine the pK_a's of Asp96 during H^+ uptake in the N $\rightarrow$ O transition (7.5) and the pK_a of the PRC in the O $\rightarrow$ bR transition (4.5). These pK_a's and the rate of H^+ transfer depend on several residues.

In this study we examined the roles of Thr205 and Ser193, the members of the chain of hydrogen bonding residues and water molecules which connect Asp85 with the extracellular surface. Mutation of Thr205 to Val increased the pK_a of the PRC in M by 2 pK units and decreased the rate of the O $\rightarrow$ bR transition (by about 30 times) which resulted in the slowing of the photocycle to a turnover rate similar to that seen in mutants with a disabled PRC (E204Q, E194Q). A similar decrease was also observed in the rate of deprotonation of Asp85 in pH jump experiments (from pH 2 to 7) for the initial bR state. These data indicate that Thr205 is critically involved in internal H^+ transfer from Asp85 to the PRC, presumably by interacting with a water molecule close to Glu204 in O.

Ser193 interacts both with Glu204 and Glu194 in the initial state. The S193A mutation at neutral pH produces an effect opposite to that of T205V: no O accumulation is observed, indicating accelerated deprotonation of Asp85. However at low pH the O $\rightarrow$ bR transition slows dramatically and becomes limiting because of the elevated pK_a of the PRC in O (>5.5 versus 4.5 in WT), which leads to the inability of the PRC to accept a proton from Asp85.

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L-11 THE EFFECT OF SURFACE MUTATION ON 'PROTON HOLE' PROPAGATION DYNAMICS IN PHOTO ACTIVATED BACTERIORHODOPSIN

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The delivery of the protons to the cytoplasmic orifice of the proton-channel is mediated by a proton-collecting antenna made of surface carboxylates that are located at the vicinity of D38 and protonate it within a few microseconds.

Fast kinetic measurements revealed that a single charge replacement, even at a location which is remote from the orifice, could modulate the rate constants of proton delivery to the orifice. Thus, when one residue is omitted from the structure, others maintain the efficiency of the process. For example, the mutant R227Q exhibits a 20-fold decrease in its rate constant for proton transfer between the proton collecting antenna and acceptor in the bulk. Yet, the protonation of the orifice (D38) was still attained within $\tau<20$ μs, and equilibration between bulk and surface was established in less than 500μs.

In contrast to the above findings, the Schiff base-bulk reaction is rather slow and a state of disequilibrium can be detected in the 10 ms$\leq$ t $\leq$200 ms time range, which is orders of magnitudes longer than the bulk surface equilibration time. The disequilibrium is characterized by the situation in which the content of the deprotonated Schiff base (measured at 415 nm) is significantly smaller than the excess of proton in the bulk (determined as protonated indicator). Based on the 'standard theory', this situation is equated with Br molecules, where the D96 had donated its proton to the Schiff base, yet failed to be reprotonated by D38 at a compatible rate. This creates a transient state of a 'proton hole' at the center of the protein, and its relaxation is through a mechanism of hole propagation from D96 towards its donor D38. The hole propagation reaction is a very fast ($\tau<1$ns), but is a low probability ($\tau\geq10$ms) event. The passage of proton between the two residues transverses a hydrophobic barrier that must be transiently solvated in order to allow the 'proton hole' on D96 to be filled. Most probably it appears during random fluctuation of the protein, where the fracture zone opens up and the water molecule permeates inside.

L-12 CELLULAR AND STRUCTURAL DETERMINANTS OF BACTERIORHODOPSIN BIOGENESIS

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Biogenesis of bacteriorhodopsin (BR) and the purple membrane in *Halobacterium salinarum* requires coordinate assembly of the bacterioopsin (BO) polypeptide, retinal, and lipid molecules. We have taken two approaches to understanding a key step in this process, assembly of the BO-retinal complex. First, we have identified cellular factors that participate in retinal biosynthesis, including genes for phytoene synthase (*crtB1*), lycopene cyclase (*crtY*), and two proteins that may function in β-carotene cleavage (*brp* and *blh*). In-frame deletion of these genes in *H. salinarum* abolishes BR and retinal production without affecting BO production. Phylogenetic analysis, coupled with gene expression data from other investigators, suggests that these genes encode components of a multi-protein complex essential for retinal production during BR biogenesis. Second, we have carried out semi-random mutagenesis to identify amino acid residues of BR that are necessary for its formation in *H. salinarum*. Mutations were targeted to regions of up to 16 amino acids in length with a strategy designed to yield an average of 5.5 substitutions per mutant. So far, 177 residues have been targeted, and more than 750 purple mutants have been sequenced. Approximately 150 of the targeted residues could be substituted without substantially affecting the yield or spectrum of BR. Of the remaining residues, 16 contact retinal, and 15 of these were intolerant to substitution or were substituted conservatively. The rest include amino acids that are far from the retinal binding pocket, such as Gly65, Trp80, Gly116, Gly125, Thr128 and Leu174. These residues are likely to play a key role in BR formation in *H. salinarum*, possibly in the assembly of the BO-retinal complex.

L-13 BACTERIORHODOPSIN AND OXIDATIVE STRESS: EXAMINING THE MEMBRANE THEORY OF AGING

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Besides its interest as a light-driven proton-pump, in previous years bacteriorhodopsin (BR) successfully serves as a model system to study the specific properties of integral membrane proteins. We have employed bacteriorhodopsin to evaluate the validity of "The free radical hypothesis of ageing" and "The membrane theory of ageing", using the peroxynitrite anion ($ONOO^-$) as reactive species. This oxidizing species is involved in pathophysiology and cellular defense against infection.

Treatment of the membrane protein bacteriorhodopsin in the purple membrane or the water soluble protein bovine serum albumin (BSA) with peroxynitrite (PN) leads to the formation of 3-nitrotyrosine (NT). As in the case of BSA there are no other modifications of amino acids detectable in BR besides NT. There is no reaction of PN with retinal according to UV-VIS-spectroscopy. The concentration of $ONOO^-$ needed to induce alterations are strikingly higher for PM as compared to BSA. In the case of PM just 0,2 % of the applied ONOO- (in absence of CO_2) react to NT (BSA: 5 %). This is most likely the consequence of the special arrangement of BR in PM. The presence of CO_2 enhances nitration of tyrosines (at higher CO_2-concentrations this effect is diminished). The rate constants of the formation and the decay of the M-intermediate are more or less halved by treatment of PM with PN in the absence of CO_2. With increasing concentrations of CO_2, the values of the rate constants approximate to those of untreated PM.

Besides investigation of trimeric BR in the PM, containing only few saturated isoprenoid lipids, experiments were performed with BR reconstituted in different lipid environments. Under the conditions chosen BR was present as mobile monomer, completely surrounded by the lipids DOPC (18:1) or DLPC (18:2). Due to lipid peroxidation the damage of BR in BR-DLPC-liposomes is augmenting until complete destruction of the chromophore and loss of protein integrity.

L-14 TWIST-SHARING ONE BOND ROTATION (TSOR) MECHANISM IN THE CIS-TRANS PHOTOISOMERIZATION OF RHODOPSIN

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Visual pigment, rhodopsin, has two important properties. One is the high photosensitivity: The photoisomerization time is as small as 200fs while the thermal isomerization time is as large as a hundred of billion sec. The ratio is as small as 10^{-23}. The other is the high specificity of *cis-trans* photoisomerization: only 11 *cis*-> all-*trans* isomerization takes place. Although much study has been done so far to resolve the mechanism of these properties, satisfactory answer was not obtained.

Recently, two important advancements were made. One is the clarification of the three dimensional structure of rhodopsin by the X-ray crystallographic study. The other is the finding of the conical intersection point between the ground and excited state by much advanced quantum chemical calculations. Then, we are ready to study the mechanism of the *cis-trans* photoisomerization by the molecular dynamics simulation. We do it. In this, we adopt Mt. Fuji potential for the twistings around C11=C12, C9=C10 and C13=C14, modeling the potential energy surfaces calculated by the quantum chemical theory.

We found that the *cis-trans* photoisomerization takes place in about 150 fs, in agreement with the experimental result. The photoisomerization is a counterclockwise one-way rotation. The isomerization is bond-specific; only C11=C12 bond is rotated. We made analysis of the *cis-trans* isomerization by substituting the retinal analogs; 9C-demethyl retinal and 13C-demethyl retinal. We also used mutant rhodopsin substituting Thr118 with Gly.

As a result, we found the followings. (1) Due to the intramolecular steric hindrance between 20-metyl and 10-H, the C12-C13 and C10-C11 bonds are twisted counterclockwise in rhodopsin, allowing only counterclockwise rotation of C11=C12 in the excited state. (2) The movement of 19-methyl in rhodopsin is blocked by the surrounding three amino acids, Thr118, Met207 and Tyr 268, prohibiting the rotation of C9=C10. Only all-*trans* form of the chromophore is obtainable as a photoproduct. (3) At the 90 degree twisting of C11=C12 in the course of photoisomerization, twisting energies of the other bonds amount to about 20 kcal/mol. (4) If the transition state for the thermal isomerization is assumed to be similar to this structure, the activation energy for the thermal isomerization around C11=C12 in rhodopsin is elevated by about 20 kcal/mol and the thermal isomerization rate is decelerated by 10^{-14} times than that of the retinal chromophore in solution, protecting photosignal from the thermal noise.

L-15 UNRAVELING THE PRIMARY EVENTS IN PHOTOEXCITED BACTERIORHODOPSIN USING STIMULATED EMISSION PUMPING

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New information concerning the photochemical dynamics of bacteriorhodopsin is obtained by impulsively stimulating emission from the reactive fluorescent state. Depletion of the excited state fluorescence leads to an equal reduction in production of later photoproducts. Accordingly, chromophores which are forced back to the ground state via emission do not continue on in the photocycle, conclusively demonstrating that the fluorescent state is a photocycle intermediate. The insensitivity of depletion dynamics to the "dump" pulse timing, throughout the fluorescent states lifetime, and the biological inactivity of the dumped population, suggest that the fluorescent state structure is constant, well defined, and significantly different than that where crossing to the ground state takes place naturally. In conjunction with conclusions from comparing the photophysics of BR with that of synthetic analogues containing "locked" retinals, present results show that large amplitude torsion around C_{13}=C_{14} is required to go between the above structures.

L-16 MOLECULAR MECHANISM OF RHODOPSIN ISOMERIZATION AND PROTEIN ACTIVATION FROM RESONANCE RAMAN SPECTROSCOPY

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Recent work from our group has included Stokes and anti-Stokes Raman studies of the picosecond structural dynamics of retinal photoisomerization in rhodopsin,[1,2] a more detailed examination of the photoreaction quantum yield in light of the current nonstationary wavepacket isomerization model,[3] and Raman and pigment mutant studies of the later intermediates that reveal a counterion switch in the rhodopsin activation process.[4,5,6] Briefly, the ps time-resolved Raman studies show that the all-trans chromophore structure in photorhodopsin is formed within 1 ps, that the photo-to-batho relaxation process is due to vibrational cooling, and that the energy storage in the primary photochemistry is complete on a picosecond time-scale.[1,2] The photochemical studies show that the quantum yield for visual photochemistry is 0.65 at 500 nm and that quantum yield is wavelength dependent, dropping by 5% as the photon energy is reduced in the red.[3] These results were predicted by our nonstationary wavepacket isomerization model for visual photochemistry. Resonance Raman spectra of the BSI, Lumi and Meta I intermediates[4,5] reveal that the $C=NH^+$ stretching mode in BSI exhibits a normal frequency and deuteration induced downshift, implying a normal counterion interaction with Glu113. Surprisingly, the $C=NH^+$ stretching mode frequency in Lumi is unusually low and shifts only 7 cm^{-1} in D_2O, suggesting a dramatic weakening of the interaction of the Schiff base group with its counterion. However, the $C=NH^+$ stretching mode in Meta I is found at 1654 cm^{-1} and exhibits a normal deuteration induced downshift. Based on these data we proposed that there is a switch of the Schiff base counterion between BSI and Meta I. The identity of the counterion in Meta I was established through detailed biochemical and spectroscopic studies of a series of Glu181 mutants.[6] In particular, E181Q pigments produce a Meta I species whose absorption is pH dependent with the same phenotype as the E113Q mutants of rhodopsin in the dark state. We conclude that there is a counterion switch in the transition from BSI through Lumi to Meta I that is facilitated by proton transfer through a H-bond network from Glu181 to Glu113. Such a counterion switch is a general feature of visual pigment activation and may be relevant for all G-protein coupled receptors.

Acknowledgement: Portions of this work was performed in collaboration with the research groups of Johan Lugtenburg, Tom Sakmar and Dave Kliger. A complete list of contributors will be found in the poster abstracts by first authors D. Pan, J. Kim and E. Yan.

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L-17 RECREATING FUNCTIONAL ANCESTRAL ARCHOSAUR VISUAL PIGMENTS

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The ancestors of the archosaurs, a major branch of the diapsid reptiles, originated more than 240 million years ago near the dawn of the Triassic Period. We employed maximum likelihood phylogenetic methods to infer the amino acid sequence of a putative ancestral archosaur visual pigment. We tested the ancestral sequence by chemically synthesizing the corresponding gene, which was then expressed in a mammalian cell line in tissue culture. The expressed artificial gene bound 11-*cis*-retinal to yield a stable photoactive pigment with a λ_{max} value of 508 nm, which is slightly red-shifted relative to that of extant vertebrate pigments. The ancestral archosaur pigment also activated the retinal G protein transducin. Our results show that ancestral genes from ancient organisms can be reconstructed *de novo* and tested for function using a combination of phylogenetic and molecular methods. We have also synthesized several other ancient ancestral opsin genes designed to elucidate the early divergence between rod and cone pigments.

L-18 THREE DISTINCT RETINAL PROTEINS EXPRESSED IN THE BRAIN OF THE PRIMITIVE CHORDATE, ASCIDIAN

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Ascidians are lower chordates, and their tadpole-like larvae share a basic body plan with vertebrates. The ascdian larva has a simple central nervous system with about 100 neurons. The brain contains an eyespot (ocellus), consisting of three lens cells, one pigment cup cell, and about 20 photoreceptor cells. To study phototransduction systems in ascidians, we have isolated and characterized three cDNAs encoding opsin family protein from larvae of the ascidian *Ciona intestinalis*. Ci-opsin1 and Ci-opsin2 are vertebrate-type opsins closely related to the retinal and pineal opsins of vertebrates. The *Ci-opsin1* mRNA is specifically expressed in the photoreceptor cells of the ocellus and the protein is localized in outer segments of the photoreceptor cells. The vertebrate opsin-type sequence and the expression pattern of *Ci-opsin1* suggest a common descent of the ascidian ocellus and the vertebrate eyes. The *Ci-opsin2* gene is not expressed in the photoreceptor cells, but in a subset of neurons in a ventral part of the brain. Ci-opsin3 is a retinal photoisomerase similar to retinochrome and mammalian RGR. In contrast to squid retinochrome and mammalian RGR, however, Ci-opsin3 is localized in the entire region of the brain as well as in motor neurons. Ci-opsin3 may play a role not only in the activity of the photoreceptors, but also in the physiological function of the central nervous system. Our results suggest that the ascidian opsins have both vertebrate-type and unique features. The latter may represent an ancestral state of the vertebrate phototransduction system.

L-19 COMPARATIVE STUDY ON THE DIVERSITY IN RHODOPSIN FAMILY

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Rhodopsins and their related proteins are divided into several subgroups on the basis of the amino acid sequence similarity. Each of them contains a retinal as a chromophore, which binds to Lys-296 (bovine rhodopsin numbering system) through a protonated Schiff base linkage. A negatively charged amino acid residue, "counterion" should be present to stabilize the protonation for visible light absorption. In vertebrate visual pigments, Glu-113 in the helix III serves as a counterion. The other pigments including invertebrate rhodopsins and retinochrome/RGR have amino acid residues different from Glu at this position and therefore, these pigments could have a counterion at a different position. Thus we tried to elucidate the counterion in these pigments by site-specific mutagenesis.

The mutational analyses of a series of retinochrome mutants revealed that Glu-181 in the extracellular IV-V loop acts as the counterion in retinochrome. Furthermore, we analyzed Glu-181 mutants of pigments other than the vertebrate rhodopsins and retinochrome. The result strongly supported the idea that Glu-181 serves as a counterion in the members of rhodopsin family other than vertebrate rhodopsins. Based on these results, the diversity of rhodopsins will be discussed from the viewpoint of counterion.

L-20 DIVERSITY AND EVOLUTION OF VERTEBRATE PHOTOTRANSDUCTION SYSTEMS

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In photoreceptor cells, light activates visual pigments consisting of a chromophore (retinal) and a protein moiety (opsin). Activated visual pigments trigger an enzymatic cascade, called phototransduction cascade, in which more than ten phototransduction proteins are participating. Two types of vertebrate photoreceptor cells, rods and cones, play roles in twilight and daylight vision, respectively. Cones are further classified into several subtypes based on their morphology and spectral sensitivity. Though the diversities of vertebrate photoreceptor cells are crucial for color discrimination and detection of light over a wider range of intensities, the molecular mechanism to characterize the photoreceptor types remains unclear. We investigated the amino acid sequences of about 50 vertebrate opsins, and found that these sequences can be classified into five fundamental subfamilies. Clear relationships were found between these subfamilies and their characteristic spectral sensitivities. In addition to opsins, we studied other phototransduction proteins. The amino acid sequences of phototransduction proteins can be classified into a few subfamilies. Even though their spectral sensitivity is considerably different, cones fundamentally share the phototransduction protein isoforms which are different from those found in rods. It is suggested that the difference in phototransduction proteins between rods and cones is responsible for their sensitivity to light. Isoforms and their selective expression may characterize individual photoreceptor cells, thus providing us with physiological functions such as color vision and daylight/twilight visions.

L-21 THE DIFFERENCE IN MOLECULAR PROPERTIES BETWEEN ROD AND CONE VISUAL PIGMENTS

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The visual transduction process in photoreceptor cells begins with photon absorption by a visual pigment. In most vertebrates, different types of visual pigments are present in the rod and cone photoreceptor cells, where they mediate vision under twilight and daylight conditions, respectively. Biochemical investigations revealed that the cone visual pigments exhibit a common motif of molecular properties different from that of the rhodopsin. Thus identification of the amino acid residue(s) responsible for the difference in molecular properties between rod and cone visual pigments is important for furthering our understanding of the functional diversity of the visual pigments.

There are at least two criteria for identifying the amino acid residues. One is based on the chemical characteristics of the amino acid residues and the other is based on the conserved nature in the primary sequences. Based on the facts that the cone visual pigments have many basic amino acid residues while the rhodopsins have acidic residues, we previously selected the amino acid positions where the amino acid residues in the cone visual pigments have an electric property different from those in the rhodopsin. Mutational experiments revealed that the glutamic acid at position 122 is one of the determinants of the molecular properties unique to rhodopsin[1]. Here we have tried to determine amino acid residue(s) from the other criterion based on the conserved nature in the primary sequences. For this purpose, we selected the amino acid positions where all the known cone visual pigments have the same amino acid residues that are different from those of the rhodopsins. Among the three positions (Val77, Gly144 and Pro189) that meet this criterion, the proline residue at position 189 is responsible for the unstable properties of chicken green opsin and meta intermediates of chicken green. From these findings, the molecular mechanism underlying the difference in molecular properties between rod and cone pigments will be discussed.

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L-22 WHY DOES NATURE FORM A PURPLE MEMBRANE?

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Bacteriorhodopsin in *Halobacterium salinarum* occurs as a two-dimensional crystal, the purple membrane, and crystallizes spontaneously independent of the cellular concentration. This indicates a high free energy of the association reaction.

Feeding deuterated glucose to halobacteria allowed to label the head groups of the purple membrane glycolipids and neutron crystallography localized three molecules inside the trimer in the lattice and three molecules between the trimers. X-ray crystallography of the trimer lipid complex identified the exact position of the inner glycolipid molecules. All residues involved in lipid-protein interaction were mutated and the mutants inspected for their ability to form purple membranes. Specifically mutations of tryptophan 80, although producing bacteriorhodopsin in standard amounts, were devoid of any purple membrane. By specific labelling and epr spectroscopy, linear dichroic studies and chemical photocross-linking experiments it was shown that by all likelihood in this mutant bacteriorhodopsin occurs as a monomer in the cell membrane. The specific proton translocation activity and the capacity for photophosphorylation was equal in the mutant and the wild type strains. A dramatic difference, however, was seen when cells were grown phototrophically, i.e. without oxygen in light. The mutant, in contrast to wild type, could not grow under these conditions for the following reason: In contrast to wild type purple membranes mutant BR in light shows after initial light adaptation a continuous bleaching of the chromophore band caused by formation of 9-cis retinal. The process occurs over the period of minutes to hours and explains why no difference in proton translocation activity or photophosphorylation activity in the intact cells is found.

Thus, it appears that nature created a protein-lipid lattice of the purple membrane to stabilize the protein in such a way that the all-trans/13-cis thermoreversible isomerization occurs with perfection and no side reaction on the scale of hours to days, which are necessary for photosynthetic growth, can occur.

L-23 STRUCTURAL DYNAMICS OF RETINAL PROTEINS: LESSONS FROM X-RAY CRYSTALLOGRAPHY AND MICROSPECTROPHOTOMETRY

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We have developed a novel concept for the crystallization of membrane proteins using highly viscous, optically transparent and by non-birefringent lipidic cubic phases[1] - the *in cubo* crystallization. Crystals grown *in cubo* yielded the high-resolution X-ray structures of bacteriorhodopsin (bR) in its ground[2] and early intermediate[3,4] states, revealing the early rearrangements that occur upon photoactivation and lead up to vectorial proton pumping. Recent crystal structures of sensory rhodopsin II (SRII) in its ground[5] and first intermediate state[6] illustrate similarities and differences to bR. Advances and limitations of intermediate trapping, or kinetic crystallography will be discussed.

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L-24 ELECTRONS VS. X-RAYS IN THE ANALYSIS OF PROTEIN CONFORMATIONAL CHANGES IN BACTERIORHODOPSIN

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Upon illumination, bacteriorhodopsin undergoes a single, large protein conformational change, associated with the switch in accessibility of its Schiff base from the extracellular to the cytoplasmic side. An atomic model for this structural change has been derived recently from electron diffraction analysis of two-dimensional crystals of the D96G/F171C/F219L triple mutant, demonstrating that the changes are principally localized to the cytoplasmic regions of helices F and G. Atomic models for light-induced conformational changes that occur in the course of, and upon, formation of the cytoplasmically "open" state have also been reported from X-ray crystallographic studies of three-dimensional crystals. The atomic models derived by X-ray crystallography do not bear a resemblance to the structural change deduced from electron crystallographic analysis, and also significantly differ from each other with regard to the nature and extent of the deduced conformational change. By combining phases obtained from direct imaging of the D96G/F171C/F219L triple mutant with amplitudes obtained from electron diffraction patterns, we demonstrate that the experimentally determined difference Fourier map describing the main protein structural change closely matches the difference map calculated from the co-ordinates derived from electron, but not X-ray crystallography. These findings establish that the helix movements that occur in two-dimensional crystals of bacteriorhodopsin under physiologically relevant conditions, as part of the mechanism of light-driven proton transport, are not observed in the three-dimensional crystals used for the X-ray crystallographic studies.

L-25 STRUCTURE AND PROPERTIES OF PHOTOINTERMEDIATE AND RELATIONSHIP TO THE PHOTOREACTION

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We have clarified that bacteriorhodopsin undergoes a large conformational change upon light absorption by low resolution X-ray diffraction. The large conformational change is also confirmed by neutron and electron diffraction. Further, we demonstrated that there are two types of structures in the photointermediates, namely M type and N type, and that the transition from the M type to the N type structure is hydration dependent. Time-resolved X-ray diffraction on wild type bR revealed the existence of these two types of structures in the real photoreaction cycle. Based on these findings, we proposed the conformation controlled conformation change model for the proton pump mechanism [1]. On the other hand, there are arguments that one conformation is sufficient to describe the proton pump mechanism.

Recently, high resolution X-ray crystal structure analysis revealed the atomic details of photointermediates. Generally, the large conformational change described by low resolution diffraction techniques is not necessarily observed in the crystal structures. We have to analyze the structural properties revealed by low resolution technique based on the crystal structures. In the talk, we will discuss the relationship between low resolution structure and the crystal structure. We also would like to discuss the validity of two types of photointermediate structures.

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L-26

HIGH RESOLUTION STRUCTURES OF INTERMEDIATE STATES OF BACTERIORHODOSIN

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The extent of conformational changes in bacteriorhodopsin (bR) and their connections with the pumping process became evident through the investigation of the M intermediate by low resolution diffraction with neutrons (1), X-rays (2), and electrons (3). Due to the new concept for crystallization of bR in the lipidic cubic phase (4) high resolution intermediate structures became available. We shall present new ground state and intermediate structures of wild-type bR. New results of the dynamacs of water molecules in bR and especially in the cytoplasmic domain will be shown.

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L-27 CRYSTALLOGRAPHIC DESCRIPTION OF THE PHOTOINTERMEDIATES OF BACTERIORHODOPSIN

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We produced the K and M_1 intermediates in bacteriorhodopsin crystals in photostationary states, at 100K with 40% yield for K, and at 210K with 60% yield for M_1, and determined their x-ray diffraction structures to 1.43 Å resolution. In K the C_{13}=C_{14} bond is rotated nearly fully to *cis* from the initial *trans* configuration, but the C_{14}-C_{15} and C_{15}=NZ bonds are partially counter-rotated. This strained geometry keeps the direction of the Schiff base N-H bond vector roughly in the extracellular direction, but the angle of its hydrogen bond with water 402, that connects it to the anionic Asp-85 and Asp-212, is not optimal. Weakening of this hydrogen-bond may account for many of the reported features of the infrared spectrum of K, and for its photoelectric signal, as well as the deprotonation of the Schiff base later in the cycle. Importantly, although 13-*cis*, the retinal does not assume the expected bent shape of this configuration. Comparison of the calculated energy of the increased angle of C_{12}-C_{13}=C_{14}, that allows this distortion, with the earlier reported calorimetric measurement of the enthalpy gain of the K state indicates that a significant part of the excess energy is conserved in the bond strain at C_{13}. In M_1 water 402 is seen to move, nearly 1 Å away from the unprotonated retinal Schiff base nitrogen. This breaks the hydrogen bond that bridges them, and initiates rearrangements of the hydrogen-bonded network of the extracellular region that develop more fully in the intermediates that follow. In the M_1 to M_2 transition, relaxation of the C_{14}-C_{15} and C_{15}=NZ torsion angles to near 180° reorients the retinylidene nitrogen from the extracellular to the cytoplasmic direction, water 402 becomes undetectable, and the side-chain of Arg-82 is strongly displaced toward the Glu-194 and Glu-204. Finally, in the M_2 to M_2' transition, correlated with release of a proton to the extracellular surface, the retinal assumes a virtually fully relaxed bent shape, and the 13-methyl group thrusts against the indole ring of Trp-182 which tilts in the cytoplasmic direction. Comparison of the structures of M_1 and M_2 reveals the principal switch in the photocycle: the change of the angle of the C_{15}=NZ-CE plane breaks the connection of the unprotonated Schiff base to the extracellular side and establishes its connection to the cytoplasmic side.

L-28

LIGHT-DRIVEN ION PUMPING AND SIGNALING IN BACTERIAL RHODOPSINS

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We report an atomic resolution structure for a sensory member of the microbial rhodopsin family, the phototaxis receptor sensory rhodopsin II (*Np*SRII) that mediates blue-light avoidance by the haloarchaeon *Natronobacterium pharaonis*. The 2.4 Å structure reveals features responsible for the 70-80 nm blue-shift of its absorption maximum relative to those of haloarchaeal transport rhodopsins, and structural differences due to its sensory, as opposed to transport, function. Multiple factors appear to account for the spectral tuning difference with respect to bacteriorhodopsin: 1) repositioning of the guanidinium group of Arg^{72}, a residue that interacts with the counterion to the retinylidene protonated Schiff base, 2) rearrangement of the protein near the retinal ring, and 3) changes in tilt and slant of the retinal polyene chain. Inspection of the surface topography reveals an exposed polar residue, Tyr^{199}, not present in bacteriorhodopsin, in the middle of the membrane bilayer. We propose this residue interacts with the adjacent helices of the cognate *Np*SRII transducer *Np*HtrII.

L-29 THE MECHANISM OF PHOTOACTIVATION OF YELLOW PROTEINS

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Photoactive yellow protein (PYP) from the purple-sulphur bacterium *Ectothiorhodospira halophila* is the photoreceptor for a blue-light avoidance response, which shows many similarities with the Archaeal sensory rhodopsins. PYP, however, contains a 4-hydroxy-cinnamic acid chromophore, and is water-soluble. Activation of PYP proceeds through light-induced *trans/cis* isomerization of the 7,8-vinyl bond of its chromophore. This configurational transition is followed by a large series of conformational alterations, which ultimately leads to a modulation of the motility machinery of the photoreceptor-producing cell.

The coumaryl chromophore of PYP is present in the anionic form in the ground state (pG) of the protein. The anionic phenolate is buried within the hydrophobic core of the protein and is stabilized via a hydrogen-bonding network, involving the amino acids Y42, T50 and (protonated) E46. Photoactivation of PYP generates an excited state with a (sub)picosecond lifetime. Relaxation of this state initiates a photocycle (Φ = 0.35), containing several transient red- and blue-shifted intermediate states. At the very short timescale, several red-shifted intermediates are formed, possibly initially via a branched pathway. The most stable of these, pR, then decays into a blue-shifted intermediate (pB), in which the chromophore is protonated and E46 is deprotonated. This deprotonated E46 generates a negative charge in the interior of the main hydrophobic core of the protein, which causes destabilization and subsequent transient partial unfolding of the protein. Insight into the molecular basis of functional activity of PYP has been obtained by making use of site-directed mutagenesis and of chemical engineering of its chromophore, in combination with *in vitro* reconstitution.

Structural characteristics of these transient photocycle intermediates of PYP have been measured with a large range of biophysical techniques. These experiments have revealed the basics of the conformational rearrangements underlying photoactivation of PYP, showing that particularly the N-terminal domain and the π-helix-containing region show the most profound conformational alterations in the signaling state. In addition, these experiments led to the conclusion that the conformation of this signaling state depends on the mesoscopic environment of the protein.

For further information see *e.g.*: Genick et al., (1997) Science 275: 1471-1475; Perman et al. (1998) Science 279: 1946-1950; Rubinstenn et al. (1998) Nature Struct. Biol. 5: 568-570; Hoff et al. (1999) Biochem. 38: 1009-1017; Xie et al. (2001) Biochem. 40: 1510-1517; Imamoto et al. (2001) Biochem. 40: 6047-6052 & Van der Horst et al. (2001) FEBS Lett. 248: 1-5. Hendriks et al. (2002) Biophys. J. 82: 1632-1643.

L-30 WHAT TRIGGERS THE PROTEIN FOLDING IN PYP_M-TO-PYP_{DARK} CONVERSION: INVESTIGATIONS USING MET100 MUTANTS

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Photoactive yellow protein (PYP) is a photoreceptor protein, which is activated upon photo-isomerization of the *p*Coumaric acid chromophore and is inactivated as the chromophore is thermally back-isomerized within a second (in PYP_M-to-PYP_{dark} conversion). Here we have investigated conformational changes of the active state PYP_M and the mechanism of the rapid thermal isomerization by analyzing mutant PYPs of Met100.

The mutation to Lys, Leu, Ala, or Glu decelerated the dark state recovery by 1 to 3 three orders of magnitude. The use of one of the mutant M100L allowed us to measure conformational changes upon illumination on the minute time scale with devices sensitive to protein conformational changes such as FTIR, CD and SAXS. The results of the measurement proved that there are substantial volume changes and the secondary structural changes as PYP_M was formed from PYP_{dark}.

Temperature dependence of the PYP_M-decay rate of each of the mutants were analyzed to find that the retardation was caused in large part by elevation of the activation enthalpy ($\Delta H^{\ddagger}$) rather the other parameters such as the activation entropy or heat capacity changes. Another effect exerted by the mutations was an up-shift of the apparent pKa of the chromophore both in PYP_{dark} and in PYP_M. The correlation between them, which may explain how the PYP_M-decay rate is regulated by the 100^{th} residue, will be discussed.

L-31 ELECTROSTATIC INTERACTIONS IN PHOTORECEPTOR ACTIVATION

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Electrostatic interactions are of paramount importance in protein folding. Here we report wealth of information regarding the structural and functional importance of electrostatic interactions in photoreceptor activation of photoactive yellow protein (PYP). PYP is a putative blue light receptor protein for bacterial vision. A variety of experimental and computational techniques were employed in our studies, including time-resolved step-scan and rapid-scan Fourier transform infrared spectroscopy, light-induced H-D exchange, site-directed mutagenesis, and *ab initio* calculations. We show that (1) protonation and neutralization of the p-coumaric acid (pCA) chromophore alone do not drive large conformational changes during the PYP photocycle, and (2) dramatic changes in electrostatic interactions due to the formation of a new buried charge, COO^- of Glu46, in a hydrophobic binding site of PYP strongly destabilize the initial structure of PYP and drive large conformational changes that transform PYP from its receptor state into its proposed signaling state, leading to PYP photoreceptor activation.

L-32 THREE DIMENSIONAL STRUCTURE OF RHODOPSIN

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After a brief review on rhodopsin, we will compare the rhodopsin structure using our refined model (1HZX) with bacteriorhodopsin (1C3W[1]), model derived from the diffraction set at 2.6 Å (1L9H[2]) and the NMR measurements (1JFP[3]). Next, we will present a working model the Meta II state to determine if the "triggering" residues responsible for the rhodopsin conformational changes could be determined. After the 11-*cis*-retinal of 1HZX[4] was converted to all-*trans* isomer, the distances determined by Farrens et al[5] were scaled to correspond to the Cα distances. A program was written to rotate helix VI about its helical axis by specified degrees. Between each rotation the structure was energy minimized with cycles of rigid body minimization of pieces of the protein and Powell minimization. Distance restraints from the scaling were applied to the structure to limit the model. It was found that the restraints were satisfied best by a helix VI rotation of about 70°. Measurement of the movement of the other helices in the model indicates the motions of helix I and II fit well with the more recent measurements of the Khorana and Hubbell laboratories[6], indicating plausibility of the model.

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L-33 STRUCTURE OF NATIVE BOVINE RHODOPSIN IN A P3(1) CRYSTAL FORM

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Rhodopsin, the pigment protein responsible for dim-light vision, is a G-protein coupled receptor that converts light absorption into the activation of a G-protein, transducin, to initiate the visual response. We have crystallised detergent-solubilised bovine rhodopsin in the native form and after chemical modifications as needles 10-40 μm in cross-section. The crystals belong to the trigonal space group $P3_1$, with two molecules of rhodopsin per asymmetric unit, related by a non-crystallographic 2-fold axis parallel to the crystallographic screw along c (needle axis). The unit cell dimensions are a = 103.8Å, c = 76.6Å for native rhodopsin, but vary over a wide range after heavy atom derivatisation, with a between 101.5 and 113.9 Å, and c between 76.6 and 79.2 Å.

Rhodopsin molecules are packed with the bundle of transmembrane helices tilted from the c-axis by about 100°. The two molecules in the asymmetric unit *form contacts along the entire length of their transmembrane helices 5 in an antiparallel orientation*, and they are stacked along the needle axis according to the 3-fold screw symmetry. Hence hydrophobic contacts are prominent at protein interfaces both along and normal to the needle axis. The best crystals of native rhodopsin in this crystal form diffracted X-rays from a microfocused synchrotron sourceto to 2.55 Å maximum resolution. The structure of bovine rhodopsin in a trigonal crystal form was determined to 2.65 Å resolution by molecular replacement followed by multi-crystal averaging, using the 2.8 Å coordinates in the tetragonal crystal form (1F88) as starting model. The largest structural differences between the two crystal forms, as well as between two independent copies occurring in each crystal form, are found on the cytoplasmic face, in loop 2, loop 3 and the C-terminus beyond helix 8. This indicates a flexibility in these segments when not interacting with G-protein or regulatory proteins.

L-34 THREE DIMENSIONAL STRUCTURE AND ACTIVATION OF RHODOPSIN

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The three dimensional structure of the GPCR, rhodopsin, has been reported, first by Palczewski, et al. (Palczewski et al., 2000) and subsequently by us (Yeagle et al., 2001). These two very similar structures reflect the ground state, or inactive state, of this GPCR. Recently, using a novel approach, it has been possible to obtain structural information on the activated state of the same GPCR, metarhodopsin II (Choi et al., 2002). This new structure shows for the first time some of the conformational changes that occur upon activation. In particular the results show the opening of a groove on the cytoplasmic face of the GPCR in the excited state that is occluded in the ground state structure. Analysis of the potential surface of this activated structure and comparison with the potential surface of the corresponding G protein, transducin, reveals a complementarity that suggests a mode of binding of transducin. Utilizing recent reports from other laboratories on putative sites of contact between the receptor and the G protein, it is now possible to describe a detailed molecular model for binding of the G protein to the cytoplasmic face of the receptor. This binding motif leads to a molecular switch by which the G protein can be activated.

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L-35 STUDIES ON STRUCTURE AND MECHANISM OF RHODOPSIN BY FT-IR AND SOLID-STATE NMR-SPECTROSCOPY

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One of our major targets is to obtain structural information at (sub)atomic resolution on ligand-binding and receptor activation of the rod visual pigment rhodopsin. Here we report on our studies using solid-state NMR spectroscopy to probe chromophore structure and chromophore-opsin interactions, and using FT-IR spectroscopy to map conformational changes accompanying photo-activation.

Uniformly ^{13}C-labeled retinal was synthetized according to a novel modular procedure. The 11-*cis* and 9-*cis* isomers were obtained following photoisomerization and HPLC purification and used to prepare rhodopsin and isorhodopsin, respectively. Using 2D homonuclear and heteronuclear correlation spectroscopy all ^{13}C and ^{1}H resonances could be identified and assigned. Comparison with model compounds yields a ligation shift, that can be interpreted in terms of changes in electronic and chemical structure of the chromophore upon occupation of the binding site, and consequently of modes of interaction with opsin. First results will be presented, that already show intriguing diferences between rhodopsin and isorhodopsin.

Mapping of conformational changes is approached by identifying peak-shifts due to site-specific mutagenesis or stable-isotope labeling in the vibrational difference spectra of photochemical transitions. Residues identified so far point, in agreement with recent evidence for the involvement of a number of water molecules, at extended H-bonded networks facilitating and possibly controlling some of the photointermediate transitions.

L-36 FLOW OF RETINOIDS IN THE VERTEBRATE RETINA

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In recent years, the mutations that cause many forms of inherited retinal degenerations have been discovered to be associated with visual cycle reactions, and the knockout mouse models of visual cycle enzymes have revealed unexpected redundant pathways in the system. RDH5 (also called as 11-*cis* RDH) is proposed to be responsible for production of 11-*cis*-retinal in the vision system, however knockout mice carrying a deletion of this gene experience uninterrupted production of 11-*cis*-retinal. A combination of bioinformatics, biochemical, and molecular biological approaches allowed us to identify novel members (ds-SDRs) of the short-chain alcohol dehydrogenase/reductase (SDR) superfamily. Likely, ds-SDR1 is involved in the production of 11-*cis*-retinal from 11-*cis*-retinol in RPE cells and ds-SDR2 in the all-*trans*-retinal formation in photoreceptors. The progress in our understanding of the visual pathway comes not only from molecular techniques. A new imaging technique using two photon excitation laser scanning microscope (TPLSM) is under development to follow the flux of retinoids noninvasively in intact mice eyes. Changes of fluorescence distribution in mouse retina are measured after an intense bleaching light. The new method will increase spatial and temporal resolution compared to the current way of analyzing retinoid in intact mouse eye. This and similar imaging techniques will allow us more detailed analyses of the retinoid flow in the vertebrate retina in native and genetically altered animalmodels.

L-37 VITAMIN A, THE VISUAL CYCLE, AND DARK ADAPTATION

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Our purpose was to examine the origin and pattern of fluorescence in the rod outer segment arising following the bleaching of an intact rod and to provide evidence that it is due to accumulation of all-*trans* retinol. Rods were isolated from dark- and light-adapted retinae of salamanders and maintained in Ringer's solution in a chamber on the stage of a microscope equipped with a high sensitivity digital CCD camera. The spatial distribution of fluorescence (excitation: 360 nm; emission: 540 nm) was measured before and at various times after either treatment with exogenous agents or a bright slit of light focused on the outer segment (520 nm), calculated to bleach >99% of the visual pigment in that region. We found that following the bleaching light, a persistent fluorescence was observed in rod outer segments. When this bleaching light was focused on the distal tip, fluorescence was restricted to this region, extending only to a minor extent into unbleached regions. Treatment of uniformly bleached cells for 30 min. with lipid vesicles (0.33 mg/ml) reduced fluorescence by 40%; subsequent treatment with 1% bovine serum albumin further reduced fluorescence intensity by an additional 25%. Administration of exogenous 100 μM all-*trans* retinal resulted in an approximate 22-fold increase in total outer segment fluorescence after 1 hour. In intact cells, fluorescence intensity was greatest at the base and least at the tip of the outer segment. In broken outer segments, the increase was only modest, and no significant gradient was observed. Treatment of intact cells with all-*trans* retinol (100 μM) also resulted in an increase in fluorescence in the outer segment. However, no gradient of intensity was observed. We conclude that the fluorescence increase is attributed to the appearance of all-*trans* retinol in rods. This fluorescence declines only following treatment with agents expected to facilitate removal of lipophilic substances from the cell. The gradient of fluorescence, greatest at the base and least at the tip of the outer segment, suggests that the ellipsoid region containing mitochondria may be the source of the reducing equivalents that fuel this reduction.

L-38 INTERACTION OF THE RGR OPSIN WITH A RETINAL CHROMOPHORE

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The known retinoid-binding proteins in the retinal pigment epithelium (RPE) and Müller cells include an atypical opsin, RPE retinal G protein-coupled receptor (RGR). This nonvisual opsin in the smooth endoplasmic reticulum is closely related in amino acid sequence to invertebrate visual pigments and retinochrome, a photoisomerase that catalyzes the conversion of all-*trans*- to 11-*cis*-retinal in squid photoreceptors. The RGR opsin is bound in the dark to all-*trans*-retinal and has absorption maxima at ~469 and ~370 nm. The all-*trans*-retinal chromophore of RGR is synthesized by a proximal all-*trans*-retinol dehydrogenase in RPE microsomal membranes. Illumination *in vitro* results in the stereospecific isomerization of the bound all-*trans*-retinal to the 11-*cis* isomer. After irradiation of RGR, the bound 11-*cis*-retinal may be converted to the alcohol by 11-*cis*-retinol dehydrogenase in the presence of NADH. RGR and the 11-*cis*-retinol dehydrogenase (RDH5) co-purify reproducibly and may be closely bound in a protein complex. The RGR opsin is involved in the production of 11-*cis*-retinal in mice and is necessary for maintaining normal steady-state levels of both 11-*cis*-retinal and rhodopsin in a light-adapted eye. These results indicate that RGR functions to regenerate 11-*cis*-retinal *in vivo* and participates in a light-dependent visual cycle. The evidence for functional interaction of a novel all-*trans*-retinol dehydrogenase, RGR opsin, and 11-*cis*-retinol dehydrogenase suggests a model for chromophore metabolism and flow of retinoids in the photic visual cycle. Mutations in human *RGR*, which is located on chromosome 10q23, are associated with cases of recessive and dominant retinitis pigmentosa.

L-39 A NOVEL ALL-*TRANS* RETINOL DEHYDROGENASE IN THE PHOTIC VISUAL CYCLE OF RETINOID METABOLISM

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In the photic visual cycle, retinal G protein-coupled receptor (RGR) isomerizes all-*trans* retinal to 11-*cis* retinal in the retinal pigment epithelium (RPE) upon illumination. It is unclear, however, how all-*trans* retinal, the substrate for RGR, is generated in the RPE, as no all-*trans* retinol dehydrogenase (atRDH) has previously been identified in the RPE. We report here the identification of a novel atRDH, RDH10, which generates all-*trans* retinal in the RPE. The full-length RDH10 cDNAs were cloned from the human, bovine and mouse. RDH10 consists of 341 amino acids and has significant sequence homology with other short-chain dehydrogenases/reductases (SDR). The human RDH10 shares 100% and 98.6% amino acid sequence identity with the bovine and mouse proteins, respectively, suggesting a highly conserved sequence during evolution. As shown by Northern blot analysis, RDH10 is specifically expressed in the RPE. The cellular localization in the RPE cells was confirmed by *in situ* hybridization and immunohistochemistry using an antibody specific to RDH10. The recombinant RDH10 expressed in COS cells generated all-*trans* retinal from all-*trans* retinol. The enzyme displayed substrate specificity for all-*trans* retinol and preferred NADP as the co-factor. RDH10 did not reduce all-*trans* retinal to all-*trans* retinol in the presence of NADPH and NADH, suggesting that it is an oxidase rather than a reductase. Western blot analysis and activity assay using cell fractions from the RPE and transfected COS cells demonstrated that RDH10 is a microsomal protein. These results indicate that RDH10 is a novel retinol oxidase which can generate all-*trans* retinal from all-*trans* retinol in the RPE and may play an important role in the photic visual cycle.

L-40 RHODOPSIN AND AGE-RELATED MACULAR DEGENERATION PIGMENTS

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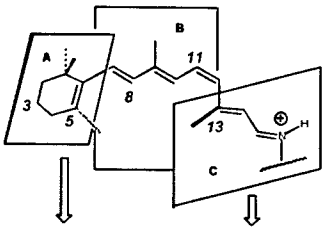
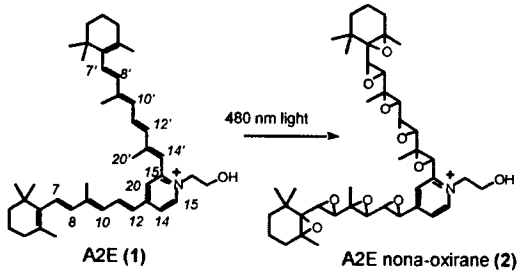
1. The sense of twist around the chromophoric 6-*s*- and 12-*s* bond. has been determined as depicted by incubation of opsin with 6-*s*-*cis*-locked and 11-*cis*-locked cyclopropyl retinoids.[1,2]



2. Studies with 11-*cis*-locked bicyclo[5.1.0]octyl retinal and other retinoids suggest the chromophore enters opsin receptor from the retinal "bottom side" (see arrows) first by filling the ring binding site, then the imine formation site, and finally the polyene site.[2]

3. A2E **1** is the major hydrophobic component of retinal pigment epithelium lipofuscin.[3] It is formed by hydrolysis of A2-PE, a precursor generated from reaction between two molecules of all-*trans*-retinal and phosphotidyl-ethanolamine. A2E mediates blue light-induced damage to RPE cells.[4] The photoproducts - oxiranes, including nona-oxirane **2**.[5] are presumably involved in apoptosis.[6]



4. Incubation of ROS with all-*trans*-retinal, 3 days at 37 °C, yields a fluorescent protein A2-Rh, i.e., a tris -A2E adduct of opsin, as suggested by its fluorescent emission and MALDI-MS.[6] Effects of antioxidants on A2E oxirane formation, passage of A2E through membranes will also be briefly discussed.[6]

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L-41 CHROMOPHORE INTERACTIONS WITH ROD AND CONE OPSINS

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The RPE65 knockout (*Rpe65*$^{-/-}$) mouse has provided a valuable model for the study of the ligand control of the activity of rod and cone opsins. This protein is abundant in the RPE and is also found in cone photoreceptors. Although the mechanism is not understood, in the *RPE*$^{-/-}$ model the photoreceptors are vitamin A deprived, as 11-*cis* retinal is not produced in the RPE. Interestingly, the RPE is in the state of hypervitaminosis as retinol collects in the form of the ester. Administration of 11-*cis* retinal can restore activity to the rods but there is no cone activity restored if the animal is one month or older at the time of dosing. The opsin of these animals has not seen its native ligand and is unusual as the activity normally associated with "bleached opsin" is not present as measured by ERGs. The opsin is also found to have a high level of phosphorylation. Administration of 11-*cis* retinal eliminates that phosphorylation and restores activity to the photoreceptors. These results suggest that rod opsin which has not previously been exposed to its ligand has a different conformation than rod opsin which is the result of bleaching. The nature of these conformational changes remains to be elucidated. The role of RPE65 in cones is not yet understood. There is a growing body of evidence that the retinoid processing system in cones differs from that of rods. Studies both on isolated red rod and red cone salamander photoreceptors and *in vitro* on recombinant salamander opsins demonstrate that the chromophore is much more loosely bound in cones that in rods. In the *RPE*$^{-/-}$ mouse, the rods remain viable up to 18 months of age but the cones degenerate within the first month. Possible mechanisms for this differential in the cone and rod viability will be discussed.

L-42 INTERNAL WATER MOLECULES OF RHODOPSINS IN ACTION

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Rhodopsins possess a positively charged retinal chromophore and negatively charged counterion(s). Internal water molecules have been presumed to play an important role to stabilize the ion-pair state inside protein. In addition, they would assist the transport of proton and chloride ion in bacteriorhodopsin and halorhodopsin, respectively. Fourier-transform infrared (FTIR) spectroscopy has observed hydrogen-bonding alterations of internal water molecules in visual and archaeal rhodopsins (1, 2). Together with the recent structural determinations of rhodopsins, water structural changes in their functional processes can be (should be) now discussed in atomic detail.

Although the frequency region was limited for the water bands under weak hydrogen bonds in the previous FTIR spectroscopy (2), we have shown the possibility to probe the strongly hydrogen-bonded water molecules by optimizing the measuring system (3). Indeed, we observed the stretching vibration of water molecule(s) associating with negative charge(s) from the spectral analysis of bacteriorhodopsin and the K intermediate (4). Such analysis was extended for *pharaonis* phoborhodopsin (5).

In my talk, the recent results of our low-temperature FTIR spectroscopy will be presented to observe internal water molecules of visual and archaeal rhodopsins during their functional processes. In particular, I like to discuss the functional role of internal water molecules in the Schiff base region of bovine rhodopsin and bacteriorhodopsin.

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L-43 PROTON TRANSFER IN BACTERIORHODOPSIN VIA PROTONATED H-BONDED NETWORKS OF INTERNAL WATER MOLECULES

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Time-resolved FTIR difference spectroscopy in combination with site directed mutagenesis is used to monitor the molecular reaction mechanisms of proteins. Based on the first fast scan studies on bacteriorhopsin a detailed proton pump model was proposed (1) which is in nice agreement with the recently published 3-D structural models. Based on succeeding step scan FTIR measurements a proton transfer via a protonated H bonded network of internal water molecules is shown for bacteriorhopsin for the release site (2) and might also present in the photosynthetic reaction center and the cytochrome c oxydase.

The proton release group consist of an $H_5O_2^+$ complex with an unusually high pK of 9.5. Complementary theoretical studies explain the high pK and localize by combination with x-ray and FTIR data its exact position between arg 82, glu 204, glu 194 close to wat 404 (3). MD calculations take into account the dynamics of internal waters and explore internal water cavities (4).

The step scan FTIR is recently for the first time applied to the photoactive yellow protein (5).

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L-44 ACTIVATION AND PHOTOCYCLING OF 11-*CIS*-LOCKED RHODOPSIN

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It is generally thought that activation of rhodopsin is initiated by the light-induced 11-*cis*→all-*trans* isomerization of the retinal chromophore. In order to provide convincing evidence for this assumption, opsin has been regenerated in the past with retinal analogues in which this isomerization is inhibited by bridging the 11,12 double bond with a ring structure. Here, we report on UV-*vis* and FTIR experiments on rhodopsin containing 6-membered ring 11-*cis* locked retinal. For the corresponding pigment, some photoreaction has been described recently involving isomerizations around the 9,10 and 13,14 double bonds (*1;2*). In the latter work, the chromophores have been classified as all-*trans* and 11-*cis* like, according to their affinities to the all-*trans* and 11-*cis* retinol dehydrogenases, respectively. FTIR experiments indicate that these isomerizations are accompanied by small protein structural changes. However, the formation of an active state similar to MII has been excluded. In earlier FTIR experiments we have shown that an active opsin state can be obtained without the chromophore by lowering the pH (*3*). Therefore, we repeated UV-*vis* and FTIR experiments with this artificial pigment at different pH values. We show that at neutral and alkaline pH mainly a photoequilibration takes place among the pigments containing the 11-*cis* like chromophores, whereas at lower pH a state is formed which has, according to FTIR difference spectra, all the signatures of the active MII state. In this state, the chromophores have the all-*trans* like geometry. In addition, it is shown that this state binds a transducin derived peptide which interacts efficiently with native MII. However, unlike native MII, this state contains a protonated Schiff base. Therefore, this state undergoes thermal back-isomerization to the inactive state on the time-scale of several minutes, the reaction rate depending on pH. Thus, a rhodopsin with a slow photocycle is described.

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L-45 STRUCTURE-FUNCTION RELATIONSHIPS IN THE EXTRACELLULAR DOMAIN OF BACTERIORHODOPSIN

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The extracellular region of bacteriorhodopsin (BR) is a complex of ionizable side chains and fixed water molecules that are organized like a chain. This multifaceted structure sustains a mutual relationship between the protonation state of the proton release group and that of Asp85, that operates during the photocycle and can be detected in the unphotolyzed protein as well.

In this communication we discuss structural/functional role of Glu and Pro residues located in the extracellular region. Results from different techniques suggest that three of the Glu side chains, Glu9, Glu194 and Glu204 are involved in cation binding sites and that the source of the released proton is a protonated water molecule linked to Glu194. On the other hand, mutation of Glu194 or Glu204 to Gln provides access of Cl^- to the protein interior. FTIR difference spectra of films show that the N intermediate is strongly prevented in these mutants in the absence of Cl^-, whereas in its presence both N-like and O' intermediates are favored.

Residues Pro8, Pro77 and Pro200 are located in the extracellular region near the Glu side chains and water molecules. Mutants of these Pro residues show altered properties, as increased values of the apparent pK_a of Asp85 (the purple-to-blue transition), or increased accessibility to hydroxylamine. Flash photolysis and FTIR difference spectra of P8W, P77G and P200G show altered photocycle kinetics and proton pumping, and favored N-like intermediate in hydrated films. These results indicate that either structurally or functionally, these Pro side chains perform important functions in the extracellular region.

L-46 PROTEORHODOPSIN AND BACTERIORHODOPSIN: SIMILARITIES AND DIFFERENCES IN STRUCTURE AND FUNCTION

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Comparisons between archaeal bacteriorhodopsin (bR) and its bacterial homolog proteorhodopsin (pR) provide important information about the conserved elements of their proton-pumping function. As expressed in *E. coli*, pR has several kDa of post-translational modifications.[1] There is heterogeneity in these modifications, resulting in the presence of two major bands on SDS-PAGE gels. These 2 forms of pR may be associated with the split C=NH vibrational band observed in resonance Raman spectra. Both components of the split band (1645, 1656 cm^{-1}) are significantly upshifted from the 1639 cm^{-1} frequency seen in bR. This indicates the presence of a significant alteration of the Schiff base counterion environment in pR, compared to bR. Other evidence supports this conclusion as well. There is a shift in the pK_a of Asp97 to 8.0 in pR from its value of 2.5 in bR. The upshifted pK_a in pR shows little sensitivity to Arg-94 mutation, or to subsequent chemical treatment of this mutant with guanidinium compounds. These differences from bR are likely due to greater physical separation between Arg94 and Asp97 in pR, in comparison to Arg82 and Asp85 in bR. Nevertheless, under conditions where an M state is formed (pH 9.5 and in the presence of phospholipids), pR exhibits fast light-activated H^+ release on the time scale of M formation. Therefore, fast light-driven H^+ release from the pR/bR family of proton pumps is not dependent on any tightly-conserved initial configuration of the residues involved in the H^+ release group. Instead, we speculate that the key conserved structural element of the H^+ release group in bR and pR is a specific H-bonded structure of the conserved arginine and surrounding residues in the M state itself. Time-resolved FTIR experiments of bR and pR continue to support assignment of a portion of the intensity in the M band at 1555 cm^{-1} to a deprotonated guanidino group.

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L-47 TIME-RESOLVED VIBRATIONAL ANALYSIS OF THE PHOTOREACTION OF PROTEORHODOPSIN FROM MARINE BACTERIOPLANKTON

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Proteorhodopsin, a homologue of archaeal bacteriorhodopsin, belongs to a newly identified family of retinal proteins from marine bacteria, which could play an important role in the energy balance of the biosphere. We cloned the cDNA sequence of proteorhodopsin by chemical gene synthesis, expressed the protein in *E. coli* cells and reconstituted the protein with lipids. Light-induced electrical measurements demonstrate that the direction of proton pumping is inverted at acidic pH. The photocycle characteristics were determined by time-resolved absorption and ATR/FT-IR spectroscopy. The pH dependence of the absorption spectrum indicates that the pK_a of the primary acceptor of the Schiff base proton (Asp-97) is 7.68. Whereas at pH > 7 an M-like intermediate is formed upon illumination, at pH 5 no M-like intermediate could be detected. As the photocycle kinetics also do not change between the acidic and alkaline state of proteorhodopsin, the only difference between these two forms is the protonation status of Asp-97. This is corroborated by FT-IR experiments, which show that major spectroscopic changes only involve the protonation status of this amino acid residue. The time-resolved FT-IR spectra are compared to resonance-Raman data to assign the chromophore vibrations and to elucidate differences in the retinal vibrations with respect to bacteriorhodopsin.

L-48 MICROBIAL RHODOPSINS: GENOME-MINING, DIVERSITY, AND STRUCTURE/ FUNCTION RELATIONSHIPS

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Photoactive 7-transmembrane helix proteins that use retinal as their chromophore were observed initially in the haloarchaea and appeared to be restricted to extreme halophilic environments. Our understanding of the abundance and diversity of the microbial rhodopsin family has been radically transformed by findings over the past 2 years. Genome sequencing of cultivated microbes as well as environmental genomics have unexpectedly revealed archaeal rhodopsin homologs in the other two domains of life as well, namely eubacteria and eukaryotes. Organisms containing these homologs inhabit such diverse environments as salt-flats, soil, freshwater, and surface and deep ocean waters, and they comprise a broad phylogenetic range of microbial life, including haloarchaea, proteobacteria, cyanobacteria, fungi, and algae. The talk will be in two parts. First will be presented the results of analysis of the new microbial rhodopsins, their expression and study, and their biophysical and functional characterization, especially of those in marine planktonic bacteria (proteorhodopsins), cyanobacteria (*Anabaena* sensory rhodopsin), and green algae (*Chlamydomonas* sensory rhodopsins). This work reveals that the newfound pigments fulfill both ion transport and sensory functions in various organisms, and use a variety of signaling mechanisms. Second, progress on crystallographic analysis of a sensory member of this family, the phototaxis receptor sensory rhodopsin II, which mediates blue-light avoidance by the haloarchaeon *Natronobacterium pharaonis*, will be presented. The structure obtained from X-ray diffraction of cubic lipid phase 3D crystals reveals key features responsible for its spectral tuning and its transducer interaction. The mechanism of SRII signaling will be discussed in terms of a unified model for transport and signaling in the widespread family of microbial rhodopsins.

L-49 STRUCTURAL INSIGHT INTO THE EARLY STEPS OF THE RECEPTOR-TRANSDUCER SIGNAL TRANSFER

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Sensory rhodopsin II (NpSRII), a seven helix repellent phototaxis receptor in the archaebacterium *Natronobacterium pharaonis* is associated with its cognate transducer (NpHtrII) a two helical membrane protein in a 2:2 complex. The receptor binding domain of the transducer comprises an N-terminally located fragment (1-114) which includes the two transmembrane helices as well as a short cytoplasmic peptide. This peptide is still capable to block the proton pump activity of NpSRII and displays a dissociation constant of about 200 nM. Excitation of the receptors retinylidene chromophore by light leads to an outward tilt of helix F which in turn induces in NpHtrII a rotational movement of the second transmembrane helix (TM2). In order to investigate the influence of the transducer on the reaction cycle of NpSRII the kinetics of the photocycle, of the helix F tilt, and of the TM2 transient mobility are analyzed by time resolved visible absorption and electron paramagnetic resonance spectroscopy. Generally, the direct environment of the receptor like lipids, detergents, or the binding of the transducer has only subtle effects on the photocycle kinetics. The outward tilt of helix F follows the kinetics of the M1→M2 transition. Similarly, the return of helix F to its original position coincides with the reformation of the ground state, i.e. the O→NpSRII transition. Similarly, TM2 changes its mobility during the M1-M2 transition. However, the back-reaction is decoupled from that of the receptor.

L-50 PHOTOCHEMICAL TRANSFORMATIONS OF NEUROSPORA RHODOPSIN, AN EUCARYOTIC HOMOLOGUE OF HALOBACTERIAL PHOTOSENSORS

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It was recently found that NOP-1, a membrane protein of *Neurospora crassa*, shows homology to haloarchaeal rhodopsins, and binds retinal after heterologous expression in *Pichia pastoris*. Various genome sequencing projects revealed many similar proteins in fungi and other eucaryotic microbes. All known rhodopsins from ascomycetes show structural prerequisites for proton pumping, possessing both primary proton donor and acceptor on helix C. At the same time, photochemistry of the *Neurospora* rhodopsin (NR) showed similarity to that of haloarchaeal sensory rhodopsin II. We studied proton pumping capability of NR using direct measurements of pH changes, flash photolysis, time-resolved FTIR spectroscopy, and site-directed mutagenesis. Proton release and uptake during the photocycle were monitored with the pH-sensitive dye, pyranine. The phenotype of the mutant NR protein, E142Q, in which the homologue of the key carboxylic acid of the light-driven proton pump bacteriorhodopsin, Asp-96, was replaced, showed that Glu-142 is not involved in reprotonation of the Schiff base. This implies that if the NR photocycle is associated with proton transport it has a low efficiency, similarly to haloarchaeal sensory rhodopsin II. FT-Raman spectroscopy revealed unexpected differences between NR and bacteriorhodopsin in the configuration of the retinal chromophore, which may contribute to the less effective reprotonation switch of NR.

L-51 TWO RHODOPSINS MEDIATE MOTILITY RESPONSES AT LOW AND HIGH INTENSITY LIGHT IN GREEN FLAGELLATED ALGAE

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Two rhodopsins, identified from cDNA sequences, function as low and high light intensity photomotility receptors in the eukaryotic alga *Chlamydomonas reinhardtii.* Each of the receptors consists of a ~300-residue seven-transmembrane helix domain with a retinal-binding pocket homologous to that of archaeal rhodopsins, followed by ~400 residues of additional membrane-associated portion. The function of the two rhodopsins (CSRA and CSRB) as the receptors is demonstrated by *in vivo* analysis of photoreceptor electrical currents and motility responses in transformants in which the expression of each of the rhodopsin genes is inhibited by double-stranded RNA interference (dsRNA*i*). The kinetics, fluence dependencies and action spectra of the photoreceptor currents differ greatly in transformants in accord with the relative amounts of the two photoreceptor pigments expressed. The data show that CSRA has an absorption maximum near 505 nm and mediates a fast photoreceptor current that saturates at high light intensity. In contrast, CSRB absorbs maximally near 470 nm and generates a slow photoreceptor current saturating at low light intensity. Our data further indicate that both phototaxis (the orientation of cells in the direction of a light beam) and photophobic responses (stop responses to an abrupt change in light intensity) are mediated by combined action of the two rhodopsins, the relative contributions of the CSRA- and CSRB-generated currents being dependent on the intensity and wavelength of the light. The saturation of the two photoreceptor currents at different light fluence levels extends the range of light intensity to which the organism can respond. The existence of two photoreceptor currents in other green algae points to a universal presence of similar bi-pigment receptor systems in this class of microorganisms.

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L-52 PROTEORHODOPSIN AND CHANNELOPSIN I: VECTORIALITY AND LIGHT ACTIVATED CHANNEL FUNCTION

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Proteorhodopsin (PR), a homologue of archaeal bacteriorhodopsin (BR) belongs to a newly identified family of retinal proteins from marine bacteria, which could play an important role in the energy balance of the biosphere. The protein was purified and reconstituted in its functional state. Optical properties were investigated by time resolved absorption and infrared spectroscopy. Pump currents were studied on a compound membrane (black lipid membranes with adsorbed proteoliposomes). The following results were obtained: The pK of Asp 97 (corresponding to Asp 85 in BR) is determined to 7.68 i.e. the absorption maximum shifts from 520 nm at pH > pK to 540 nm at pH < 7.0. At pH > pK of Asp 97 an M intermediate is detectable whereas at pH < pK no M in the acidic form of PR is visible. The photocycle kinetics do not change. This is corroborated by FTIR experiments, where only major changes occur due to the protonation status of Asp 97. The electrical measurements show that PR is a light driven proton pump. The vectoriality depends on the protonation status of Asp 97. At low pH PR is an inwardly directed pump, excited by one photon, whereas at pH > pK PR is an outwardly directed pump activated by two photons. We conclude that the protonation state of Asp 97 determines the vectoriality of PR.

Channelopsin (Chop I) occurs in the eye spot of the green algae Chlamydomonas reinhardtii. Its function is probably involved in the phototactic behavior of the cell. The hydrophobic core of the protein shows homology to BR. Although the overall homology is low, several aminoacids are conserved, that define the retinal binding site (K 296) and the H^+ binding network in BR. Chop I was expressed in the plasma membrane of Xenopus laevis oocytes. The whole protein as well as the putative 7 α helices part show light induced channel activity. The proton current follows the electrochemical gradient and is directly dependent on the concentration of protons. From the kinetics of the photocurrents, it is derived that one or several intermediates of a putative photocycle allow the channel activity of Chop I.

L-53 THE MECHANISM OF RHODOPSIN ACTIVATION AS REVEALED BY TIME-RESOLVED ABSORPTION STUDIES

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Over the years we have used time-resolved spectroscopic measurements to monitor rhodopsin photolysis intermediates on time scales from nanoseconds to seconds at near physiological temperatures. The results have revealed two additional intermediates which must appear after photoexcitation *in vivo* which do not appear in low temperature trapping experiments. At physiological temperatures bathorhodopsin (Batho) first decays on the tens of nanoseconds time scale into equilibrium with a blue absorbing species (BSI) which subsequently decays to lumirhodopsin (Lumi). Another difference was seen at physiological temperatures in the Lumi decay pathway where, beside the low temperature path to metarhodopsin I (Meta I_{480}) a second branch opens directly from Lumi to a new, deprotonated Schiff base intermediate, Meta I_{380}. Interestingly, as temperature is increased to be closer to physiological temperature the Meta I_{480} branch decreases and Meta II forms primarily through Meta I_{380}.

While spectral measurements on rhodopsin intermediates can determine a sequence of intermediates involved in rhodopsin activation, other means are needed to specify the structural nature of those intermediates. To this end we have studied a number of artificial rhodopsins in which the native retinal chromophores were synthetically modified. This has provided valuable information about which transformations of intermediates involve chromophore-localized interactions and which involve interactions with protein residues. More recent studies of rhodopsin variants have also provided insights into structural features of photolysis intermediates as well as clues regarding proton transfer reactions involved in the activation process. Results from these studies, combined with new structural information from the crystal structure of rhodopsin have yielded an increasingly detailed picture of the mechanism of rhodopsin activation.

L-54 RHODOPSIN ACTIVATION AS SEEN THROUGH THE EYE OF A MICROELECTRODE

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Charge redistribution is associated with rhodopsin conformational activation on a μsec-to-msec time scale concurrent with the $Lumi_{478} \Leftrightarrow Meta\text{-}I_{480} \Leftrightarrow Metarhodopsin\text{-}II_{380}$ spectral state equilibria. Electrical events are measured as the transmembrane R_2 early receptor current (ERC). Here we investigate three sources of energy that could modulate late conformational activation of rhodopsin - the transmembrane electrostatic field and the proton chemical potential on both the cytoplasmic and extracellular faces of the membrane. The msec-order outward R_2 component of the ERC of normal human rhodopsin senses the transmembrane electrical field (e-fold/147 mV) over a range of physiological potentials found in vertebrate photoreceptors. Depolarizing potentials (from zero imposed field) enhance the quantity of R_2 charge while hyperpolarizing potentials decrease R_2. The entirety of R_2 charge motion senses the field indicating that the electrostatic events probed by the ERC take place with absolute respect to the membrane dielectric. Intracellular pH (clamp range: pH_i 5.0-8.5) titrates the bulk (75%) of R_2 charge motion. R_2 charge increases with acidification and decreases with alkalinization (pK_a = 6.3) consistent with the known pH sensitivity of the spectral Meta-I $\Leftrightarrow$ Meta-II equilibrium. The remaining outward R_2 charge motion at alkaline pH_i has fast kinetics consistent with the rate of spectral Meta-IIa formation. The Hill coefficient (1.2) and strong dependence of R_2 relaxation rate on pH_i suggest that a significant part of the slow phase of R_2 reflects net uptake of a single proton from the cytoplasm. Extracellular pH (range: pH_o 4.0 - 9.0) has a marked impact on both the magnitude and polarity of R_2 charge but not its kinetics. Alkalinization increases while acidification decreases outward charge (pK_a = 7.0) and eventually promotes charge reversal (pK_a ~ 4.5). These studies demonstrate that R_2 ERC charge motion is a sensitive index of the late electrostatic events during rhodopsin activation. At least three proton titratable sidechains regulate R_2 charge, and proton exchange processes occur at both membrane faces before or during rhodopsin activation. These findings may have important functional implications for normal rod photoreceptor design and physiology.

L-55 METABOLIC VS. LIGHT-DEPENDENT DEACTIVATION OF VERTEBRATE RHODOPSIN

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After retinal 11-*cis*/all-*trans* isomerization, rhodopsin forms the active Meta II intermediate, in which the Schiff base linkage to Lys^{296} is intact but deprotonated. Release of the photolyzed all-*trans*-retinal (*atr*) from the chromophore pocket in rhodopsin is mandatory for eventual deactivation and regeneration of the pigment with metabolically supplied 11-*cis*-retinal. Our recent data show that the photolyzed *atr* is released through an active *atr*-opsin complex, in which the chromophore is non-covalently bound to a second binding site. During the decay reaction, neither the *atr* in this complex nor an excess of *atr* inhibit regeneration of rhodopsin with 11-*cis*-retinal. In parallel to *atr*-opsin, a "true" Meta III intermediate (ca. 30 %) is formed, in which the *atr* is retained in the retinal pocket, thus blocking regeneration (Schädel et al., see poster).

Meta III can be photoconverted to Meta II. Inversely, Meta II is deactivated by light, forming a Meta III-like product. This product (P_{500}) is different from rhodopsin or isorhodopsin by the following observations: i) the fingerprint of 11-*cis*-retinal is not present in the infrared spectra; ii) extraction of retinals from P_{500} predominantly yields all-*trans*-retinal (Bartl et al., see poster).

Deactivation of Meta II by retinal release and photolysis is preceded by rhodopsin kinase mediated phosphorylation and tight binding of arrestin. However, under dim light conditions, virtually all arrestin is in the rod inner segment and the splice variant p^{44} ($Arr^{1\text{-}370A}$) is the stop protein responsible for receptor deactivation. $Arr^{1\text{-}370A}$ is membrane bound in the dark by interaction with inactive phosphorylated rhodopsin or opsin. The data indicate a mechanism in which $Arr^{1\text{-}370A}$ is handed over from inactive to active phosphorylated rhodopsin (Schröder et al., see poster).

Another new aspect of rod deactivation is the translocation of the G-protein Gt by light-dependent bi-directional exchange through the cilium. This effect is regulated by Ca^{2+} dependent interaction of Gt with the cytoskeletal protein centrin1 (Pulvermüller et al., see poster).

L-56 BIOCHEMISTRY UNDERLYING UNIFORMITY OF ROD SINGLE PHOTON RESPONSES

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We find metarhodopsin II interaction with G protein and arrestin to decrease and increase respectively with increasing phosphorylation. Moreover, we find rhodopsin multiple phosphorylation to be anti-cooperative. Together with phosphorylation-dependent changes in kinetics, these properties suggest a mechanism of R* quench that obviates unreliable exponential decay statistics of single molecule inactivation and allows most of the Gaussian uniformity of rod single photon responses to be explained. Computer modeling of this mechanism shows response properties closely resembling those of electrical recordings from single rods.

L-57 MAPPING INTERACTIONS BETWEEN CARBOXYL-TERMINAL PEPTIDES OF THE α-SUBUNIT OF TRANSDUCIN AND A FUNCTIONAL MIMIC OF LIGHT-ACTIVATED RHODOPSIN

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Although a high-resolution crystal structure for the ground state of rhodopsin is now available (1), portions of the cytoplasmic surface are not well resolved and the structural basis for the interaction of the cytoplasmic loops with the retinal G-protein transducin (G_t) is still unknown. In an attempt to address these questions, the ability of various $G_t\alpha$-subunit ($G_{t\alpha}$) carboxyl-terminal peptides to bind to a light-activated rhodopsin mimic (2) composed of the second and third cytoplasmic loops of bovine opsin fused to a thioredoxin scaffold (HPTRX/CDEF) was examined by isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR) spectroscopy. ITC results show that an eleven amino acid peptide corresponding to the carboxyl-terminus of $G_{t\alpha}$ (GtP), as well as two peptide analogs (HAP1 and HAP2), bind to HPTRX/CDEF with micromolar-binding constants determined when fit using a single-site binding model. NMR titration experiments confirm GtP and HAP1 binding to HPTRX/CDEF and indicate that both peptides undergo small, but significant structural changes in the bound state. Comparison of the NOESY spectra of GtP and HAP1 free and in complex with HPTRX/CDEF suggest that this light-activated rhodopsin mimic does not discriminate between distinct conformations of the $G_{t\alpha}$ carboxyl-terminal peptides. HPTRX/CDEF may instead differentially interact with specific amino acid side chains that comprise the $G_{t\alpha}$ binding surface. General methods described here may be applicable to the structural analysis of G protein interactions with other GPCRs.

1. Palczewski et al. (2000) *Science* **289**, 739.
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Supported by grants from Research Corporation (to D.M.B.) and NIH (EY11112 and EY13286 to K.D.R.).

L-58 CONFORMATIONAL CHANGES IN RHODOPSINS: AN INVESTIGATION WITH TIME-RESOLVED FLUORESCENCE DEPOLARIZATION AND ABSORPTION SPECTROSCOPY

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The understanding of dynamics and conformational changes of protein surface segments, especially of the interhelical surface segments (loops) of membrane proteins and their degrees of flexibility is very important in view of their role in biological function. Both in bacteriorhodopsin (bR) and rhodopsin, major conformational changes were detected in the loop connecting helix E and F (helix V and VI) and at the cytoplasmic end of helix G (helix VII and VIII in rhodopsin). The exact nature of the molecular mechanisms underlying these conformational changes, their magnitude and the occurrence during the time course of the photoreactions is still unclear. In this study we used time-resolved fluorescence depolarization in combination with site-directed fluorescence labeling to investigate the diffusional dynamics and conformational changes on the surface of the two retinal proteins. The detailed dissection of the physical parameters effecting the conformation of protein surface segments in the inactive state allows the elucidation of the mechanisms that underlie these changes. The functional relevance of the surface potential based switch in the EF-loop of bR is supported by the finding of a transient surface charge change during the M to N transition in the bR photocycle. In the visual pigment rhodopsin the midpoint of the pH-dependent transition of helix VIII between two conformational states correlates with the midpoint of the pH dependent equilibrium between the activated Meta II and the inactive Meta I state of rhodopsin. Lowering the proton concentration leads to an almost unconstraint diffusional motion of the helix VIII on the membrane surface. However, the coincidence of the pH dependency of that transition with the pH dependency of the Meta I/Meta II equilibrium implies that the alkaline "unlock" of helix VIII in the dark state possibly prevents the activation of rhodopsin. Vice versa, a stabilized conformation of helix VIII might be a prerequisite for the formation of the active Meta II state.

L-59 INSIGHTS INTO THE ROLE OF THE "RETINAL PLUG"

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The rhodopsin crystal structure (Palczewski *et al.,* 2000) reveals that intradiscal loop E-2 forms a structure which covers the 11-*cis* retinal, forming a sort of "retinal plug ". What role does the structure of loop E-2 play in the stability and function of rhodopsin? Does it help provide a place for retinal to bind, or is the E-2 loop structure induced by retinal binding? We have begun to address these questions and recently noticed that the ends of loop E-2 appear to be held together by an ion-pair formed between residues R177 and D190. Our structure/function studies indicate this ion-pair is important for the folding and stability of dark-state rhodopsin (interestingly, mutations at D190 have previously been identified in some *retinitis pigmentosa* patients). Some mutants at R177/D190 do not appear to regenerate with 11-*cis* retinal. Those mutants that do regenerate appear wild-type like in almost all aspects except they are very unstable in their dark-state form. Arrhenius analysis shows that although these mutants denature much more rapidly at any given temperature, the activation energies for their thermal denaturation are almost identical to wild-type rhodopsin. We will present these results, discuss our recent experiments designed to determine why the loop E-2 mutants destabilize rhodopsin, and speculate on a possible role for the loop E-2"retinal plug".

L-60 A STRUCTURAL BASIS FOR A COMMON MECHANISM OF ACTIVATION IN G-PROTEIN COUPLED RECEPTORS

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1. Rhodopsin contains a conserved disulfide bond between Cys110 and Cys187.
2. Mutations such as those found in retinitis pigmentosa and deletions in any part of the intradiscal (ID) domain cause partial or total misfolding of the expressed opsin, misfolding resulting in loss of ability to bind 11-*cis*-retinal. Thus, *In vivo* folding of rhodopsin involves the formation of a tertiary structure in the ID domain comprising the N-terminal tail and the three polypeptide intradiscal loops. The structure is locked in by the formation of the conserved disulfide bond.
3. Misfolding is due to the formation of an abnormal disulfide bond, characterized by mass spectrometry to be between Cys185 and Cys187.
4. Retinitis pigmentosa mutations occurring in the transmembrane (TM) domain also cause misfolding in the ID domain via formation of the Cys185-Cys187 disulfide bond. Therefore, packing of the helices to form the TM domain and folding to form the tertiary structure in the ID domain are coupled.
5. Coupling between the two, ID and TM, domains is hypothesized to determine the helix movements and subsequent conformational change involved in receptor activation.
6. Because a conserved disulfide bond between cysteines at positions equivalent to Cys110 and Cys187 in rhodopsin is found in most of the G-protein coupled receptors, the above findings with rhodopsin lead to a hypothesis for a common structural basis for receptor activation in GPCR.

ABSTRACTS OF POSTERS

P-1 19, 19 ETHANO- AND 20, 20 ETHANO ISORHODOPSIN

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The Novel 19, 19 Ethano 9-Z- and 20, 20 Ethano 9-Z Retinals were prepared via an organic total synthetic scheme. These Retinals have a cyclopropyl group either on position 9 or on position 13 in the retinal skeleton. Their incubation with bovine opsin resulted in the new isorhodopsin systems with λ_{max} 502nm and 496nm respectively. The synthetic and biochemical aspects are discussed.

P-2 THE SYNTHESIS OF SITE-DIRECTED ^{13}C LABELED ALIPHATIC AMINO ACIDS

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Schemes have been developed to synthesize L-valine, L-isoleucine and L-leucine labeled with carbon-13 and nitrogen-15 at each position or combination of positions. The synthetic schemes allow complete control over site-directed isotope incorporation, including the distinction between the prochiral methyl groups of valine and leucine. 4(S)-^{13}C L-valine and 5(S)-^{13}C L-leucine could be prepared in good yields. The labeled amino acids are to be incorporated in rhodopsin and other (retinal) proteins and used in the search for interactions between alkyl and aromatic sidechains and their influence on protein structure. Recent work has demonstrated a similar interaction between rhodopsin's phe 202/208 and the ionone methyls of retinal.

P-3 INVESTIGATION A SPECTRAL PROPERTIES OF THE CROWN-ETHER CONTAINING RETINALS AND BACTERIORHODOPSIN ANALOGS

[1]Khodonov A. A., [1]Shevyakov S. V., [1]Lukin A. Yu., [1]Mironova E. V., [1]Shvets V. I., [1]Demina O. V., [2]Khitrina L. V., [3]Gromov S. P., [4]VedernikovA. I., [4]Barachevsky V. A., [4]Strokach Yu. P., [4]Alfimov M. V., [5]Lugtenburg J.

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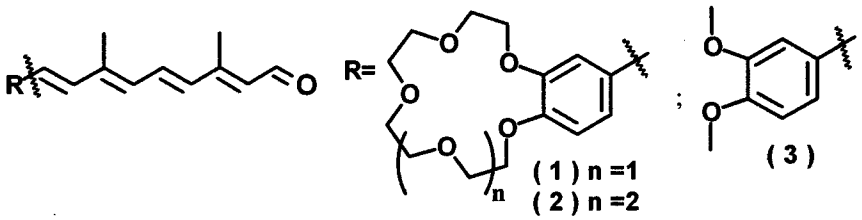
During last twenty years the design of a new synthetic methods for the introduction of crown ether moieties in the different type molecules is under extensive investigation. Recently, we reported an effective approaches for the preparation procedure [1] of new crown ether-containing polyenes **1-2** and reference compound **3**, related to Vitamin A, for the study of their photochemical properties, as well as ion-binding capability and spectral behavior of its complexes with bacterioopsin. Spectral and photochemical properties for the new retinal analogs and its bacteriorhodopsin complexes are described.



Acknowledgment

This work was supported by RFBR (projects N 01-03-32078, N 02-03-06555, N 02-03-32321) and INTAS 01-0267 grants.

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P-4 RECOGNITION AND BIOLOGICALLY RELEVANT CONFORMATION OF THE RHODOPSIN CHROMOPHORE

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The ligand of bovine visual pigment rhodopsin (Rh), a G-protein coupled receptor, is 11-*cis*-retinal. The twisted structure of the retinal around the 6-*s*-bond and 12-*s*-bond plays a key role in regulating the absorption maxima of rhodopsin. The twists also have a direct bearing in regulating the movements of the chromophore at the initial 11-*cis* to *trans* photoisomerization, which lead to conformational changes in the protein that induce the visual transduction process.

In order to determine the conformation of the chromophore, especially the absolute sense of twist around 12-*s*-bond, we used a pair of enantiomers of 11-*cis*-locked cyclopropyl retinal (retCPr) which is structurally locked with cyclopropyl and cycloheptyl rings at the 11,12-position [1,2]. Only one of the enantiomers bound to opsin, but not the other enantiomer. This clear selectivity suggested that the conformation of the bound enantiomer was the relevant conformation for the chromophore of rhodopsin. We investigated the solution conformation of retCPr by NMR, CD and molecular modeling calculations, and clarified the conformation of the bound retCPr, and the twists around the 12 -*s*-bond and the 6-*s*-bond [3]. Based on the conformation and spectroscopic data of the Rh analog and the kinetics of the binding, and by taking into account previous incorporation data, we propose the biologically relevant conformation of the chromophore.

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P-5 SPECTROSCOPIC STUDIES OF A2E INTERACTIONS WITH MEMBRANES

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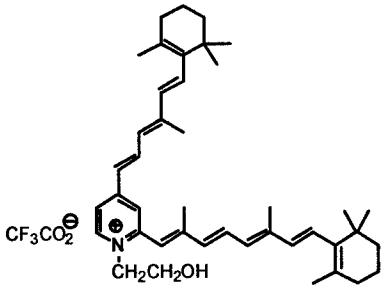
A2E, a pyridinium bisretinoid, is a prominent component of lipofuscin, the fluorescent pigments that are deposited in retinal pigment epithelial (RPE) cells with age. Elevated intracellular A2E concentrations can induce cell death and the demise of RPE in some retinal disorders such as age-related macular degeneration (AMD) is a critical component of the disease process. The amphiphilic structure of the A2E causes it to aggregate in various solvents and to disrupt cell membranes, which is likely to contribute to the damaging effects of A2E. To further elucidate the interaction of A2E with membranes and to understand the mechanism by which A2E disrupts membranes, we have undertaken NMR and fluorescence kinetic experiments using unilamellar vesicles as model systems. ^{23}Na-NMR aided by dysprosium(III) tripolyphosphate, a paramagnetic shift reagent, has been utilized as a non-invasive method to study sodium movement across the lipid bilayer. Additionally, to take advantage of the higher sensitivity of fluorescence over NMR, preliminary studies using fluorescence kinetics were carried out. Phosphatidyl choline (PC) vesicles were prepared for these initial studies, as well as vesicles incorporating a mixture of PC (50%), phosphatidyl ethanolamine (40%) and phosphatidyl serine (10%), which is a mimic of the RPE cell membrane. A2E-facilitated ion conduction across the lipid bilayer was observed in both the NMR and fluorescence experiments.

A2E



P-6 STEREOSELECTIVE SYNTHESIS OF 11-*cis*-RETINOIDS WITH DELETIONS OF THE CYCLOHEXENYL RING METHYL GROUPS

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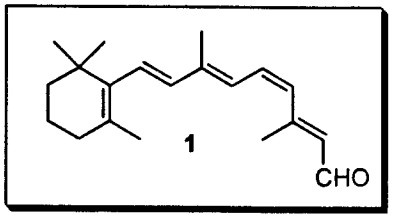
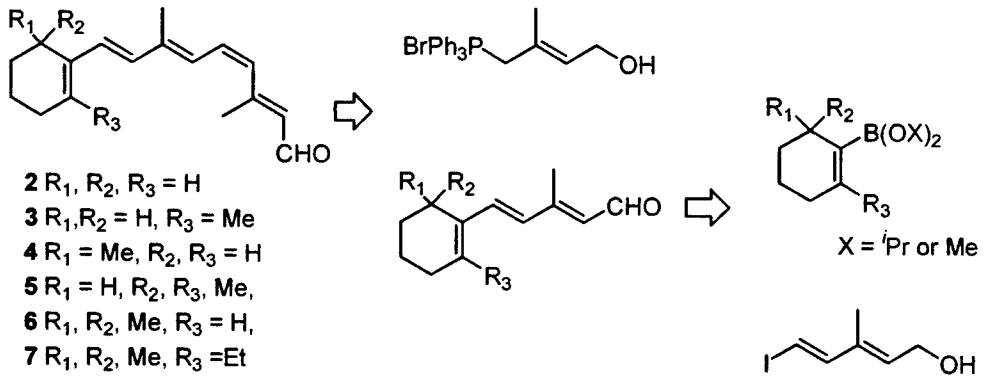
The protonated Schiff base of 11-*cis*-retinal is the light-sensitive chromophore of rodopsin, responsible for dim-light vision of vertebrates. Photochemical isomerization of **1** to the all-*trans* isomer triggers the visual process. To understand this process at the molecular level, the conformation of the retinal chromophore and its interaction with neighbour groups inside the protein-binding pocket have been studied by a variety of methods and with different synthetic retinals.

In order to understand the effect on the visual cycle of structural midifications in the cyclohexenyl ring region, we have completed the stereoselective synthesis of several demethyl analogs (**2-7**) by Suzuki coupling of an "in situ" generated cyclohexenyl boronate and an iodide to construct the C_6-C_7 bond[1]. A stereoselective Wittig[2] condensation was used to control the C_{11}-C_{12} configuration.





2 R_1, R_2, R_3 = H
3 R_1,R_2 = H, R_3 = Me
4 R_1 = Me, R_2, R_3 = H
5 R_1 = H, R_2, R_3, Me,
6 R_1, R_2, Me, R_3 = H,
7 R_1, R_2, Me, R_3 =Et

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P-7 STEREOSELECTIVE SYNTHESES OF RING-OXIDIZED RETINOIDS

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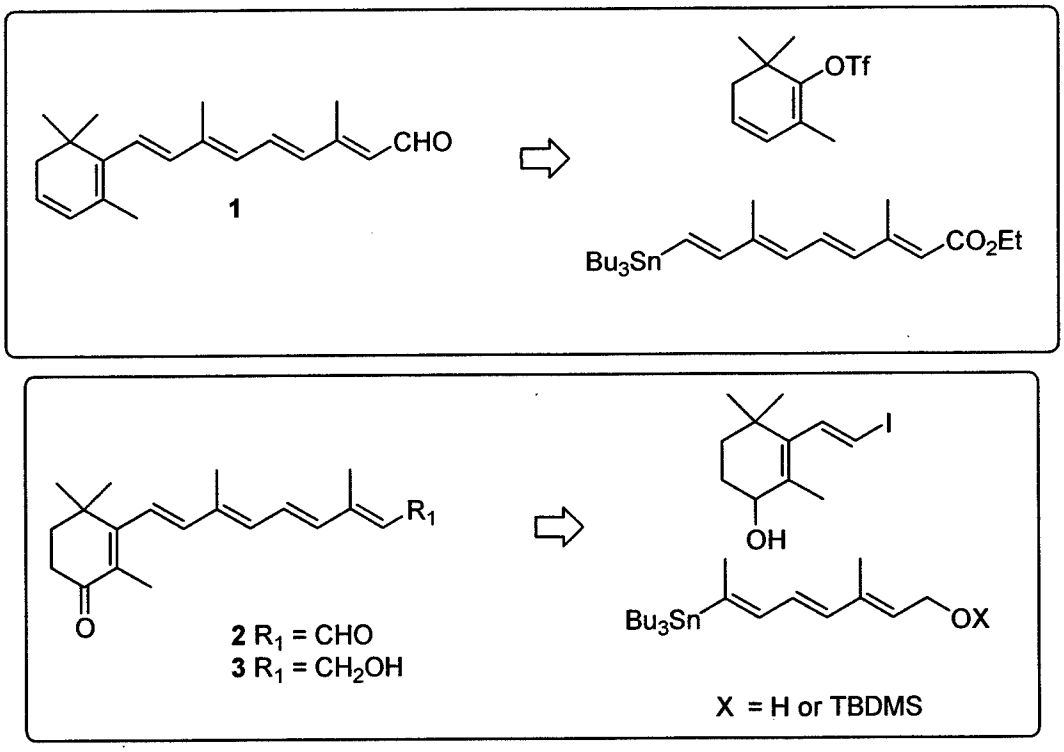
The term retinoids comprises the natural vitamin A derivatives such as retinal and retinoic acid as well as the large number of synthetic analogs prepared since the late 1960s. Formally the retinoid molecule consists of three main sections: a lipophilic part at one end, connected via a polyunsaturated chain as a spacer to a polar group at the other end of the molecule.

Based on our previous model studies[1], we will describe stereoselective syntheses of ring-oxidized retinoids, namely didehidroretinal **1**, 4-oxoretinal **2** and 4-oxoretinol **3** that involves the Stille coupling of an alectrophile (iodide or triflate) and a stereodefined polyenylstannane catalysed by transition metals, to construct a C_{sp2}-C_{sp2} single bond (C_6-C_7 or C_8-C_9) of the polyene side-chain.



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P-8 COORDINATE REGULATION OF BACTERIORHODOPSIN MEDIATED PHOTOTROPHY AND ARGININE FERMENTATION IN *HALOBACTERIUM SP.* ANALYSED BY A GLOBAL SYSTEMS APPROACH

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The extremely halophilic archaeon *Halobacterium NRC-1* can switch from aerobic organotrophy (energy from organic compounds) to anaerobic phototrophy (energy from light) by induction of bacteriorhodopsin (bR). bR, a 1:1 complex of bacterioopsin (Bop) and retinal, is a light-driven proton pump assembled as a two-dimensional lattice called the purple membrane. A light and redox-sensing transcription regulator, Bat, regulates critical genes encoding the biogenesis of the purple membrane. To better understand the regulatory network underlying this physiological switch, we report a systems approach employing global mRNA and protein analyses of four strains of *Halobacterium sp.*: the wildtype, *NRC-1* and three genetically perturbed strains, *S9* (*bat*+), a purple membrane overproducer, and two purple membrane deficient strains, *SD23* (a *bop* knockout), and *SD20* (a *bat* knockout). The integrated DNA microarray and proteomic data reveal the co-ordinated co-regulation of several interconnected biochemical pathways including isoprenoid biosynthesis, arginine metabolism, nucleotide synthesis, and carbohydrate metabolism as well as purple membrane biogenesis. In addition to the UAS motif, the Bat binding site in purple membrane regulon (set of genes regulated by the same transcription factor) promoters, we identified one additional highly conserved putative transcription factor-binding site upstream to the genes encoding arginine metabolism. This factor participates in a regulatory network including Bat to maintain a balance in ATP synthesis under anaerobic conditions via bR mediated phototrophy and arginine fermentation.

P-9 THE LIPID/BR STOICHIOMETRIES OF THE PURPLE MEMBRANE

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The lipid/protein stoichiometries of the purple membrane of Halobacterium salinarum have been obtained by a combination of ^{31}P- and ^{1}H-NMR analyses of the lipid extract. In total 10 lipid molecules per retinal were found to be present in the purple membrane lipid extract: 2-3 molecules of phosphatidylglycerophosphate methyl ester, 3 of glycolipid sulfate, 1 of phosphatidylglycerol, 1 of archaeal glycocardiolipin, 2 of squalene plus minor amounts of phosphatidylglycerosulfate and bisphosphatidylglycerol (archaeal cardiolipin) and a negligible amount of vitamin MK8. The novel data of the present study are necessary to identify the lipids in the electron density map, and to shed light on the structural relationships of the lipid and protein components of the purple membrane.

P-10 GRAFTING SEGMENTS FROM THE EXTRACELLULAR SURFACE OF CCR5 ONTO THE TRANSMEMBRANE HELICES OF BACTERIORHODOPSIN CONFERS HIV-1 CORECEPTOR ACTIVITY AND ALTERS THE FOLDING PATHWAY

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Components of the extracellular surface of CCR5 interact with certain macrophage-tropic strains of human immunodeficiency virus type 1 in a CD4-dependent manner to mediate viral fusion and entry. In order to mimic the viral interacting site(s) on CCR5, the amino-terminal and extracellular loop segments have been linked in tandem to form a series of contiguous polypeptides, or grafted onto a seven transmembrane bacteriorhodopsin (bR) scaffold either singly, or in combination, to produce a set of CCR5/bR chimeras. The CCR5 extracellular surface polypeptides and CCR5/bR chimeras were examined for stable expression, cellular localization, and/or retinal binding and CD4 dependent, JRFL gp160-mediated coreceptor activities (1). The chimera studies identified regions in CCR5 that contribute toward coreceptor activity, revealed how various combinations of extracellular segments influence folding and assembly, and highlighted a role for retinal in conferring coreceptor function. Mutational analysis of functional chimeras confirmed the specificity of the interactions with gp160.

Grafting the amino-terminal segment of CCR5, as well as the entire CCR5 extracellular surface, onto the bR transmembrane scaffold renders a chimera that is capable of modulating CD4-dependent, gp160-mediated cell fusion. Enhanced coreceptor function accompanying occupancy of the bR retinal-binding pocket may reflect structural rearrangements in the transmembrane helices that are transmitted to the CCR5 extracellular surface. The establishment of specific interactions between appended CCR5 extracellular segments and assembly of the seven transmembrane bR scaffold appears to be a cooperative process.

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Supported by NIH grants EY13286 (to K. D. R.), GM56445 (to H. L.), and Program Project grant GM39589 (to D. D. O. and T. T. S.).

P-11 HOFMEISTER-EFFECTS ON BACTERIORHODOPSIN

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Anions at moderate and high concentrations (above 50-100 mM) control the aggregation state of proteins, as well as their structure and dynamics in a correlated manner ("Hofmeister-effects"). Although the exact action mechanism is not fully understood, many experts agree that the effects are mediated by the altered structure of water in the presence of anions. Chaotropic ions ("water structure breakers") generally tend to loosen protein structures, while kosmotrops ("water structure makers") usually stabilize them. We have recently introduced a general concept for the interpretation of Hofmeister-effects based on salt-induced changes in protein free energy flucuations, that can formally account for a row of phenomena. The question how environmental fluctuations can control protein dynamics will be discussed on the basis of experiments carried out on the simplest proton pumping protein, bacteriorhodopsin.

P-12 SWITCHING BACTERIORHODOPSIN OFF WITH ANESTHETICS: STRUCTURAL FEATURES

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In the presence of halogenated general anesthetics, membraneous bacteriorhodopsin exists as an acid-base equilibrium between two forms, bR_{570} and bR_{480}. From the functional point of view, it is a real equilibrium between an active and an inactive form. From the structural point of view, the transition is accompanied by some irreversible changes. For instance, upon purple membrane addition with anesthetic, the cristalline structure of bR is definitively lost while its trimeric structure immediately recovers when the anesthetic is removed. On a smaller scale, minute secondary structural changes also occur as indicated by FTIR and isotopic exchange measurements. In order to get some image of this structural equilibrium, molecular dynamic simulation was performed on a bR trimer constructed in its natural lipid environment and the effect of penetrating anesthetic was observed on both sides of the trimer. Results show structural changes that are quite small but consistent with those observed experimentally.

P-13 X-RAY CRYSTALLOGRAPHIC STUDY OF THE PRIMARY PHOTOREACTION OF BACTERIORHODOPSIN

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Bacteriorhodopsin, the sole membrane protein of the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump. A three-dimensional crystal of bacteriorhodopsin, which was prepared by the membrane fusion method, was used to investigate structural changes in the primary photoreaction. It was observed that when a frozen crystal was exposed to synchrotron x-ray radiation, an approximately half fraction of the proteins were rapidly converted into an orange species exhibiting absorption peaks at 450, 478 and 510 nm. The rest fraction of protein retained the normal photochemical activity until Asp85 in the active site was decarboxlyated by a higher flux of x-ray radiation. The procedure of diffraction measurement was improved so as to minimize the effects of these radiation damages and determine the true structural change associated with the primary photoreaction. Our structural model of the K intermediate indicates that the Schiff base linkage and the adjacent bonds in the polyene chain of retinal are largely twisted so that the Schiff base nitrogen atom still interacts with a water molecule located near Asp85. With respect to the other part of protein, no appreciable displacement is induced in the primary photoreaction.

P-14 X-RAY CRYSTALLOGRAPHIC STUDY OF ARCHAERHODOPSIN

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Archaerhodopsin (aR) is a light-driven proton pump found in an extremely halophilic archaebacterium (*Halorubrum sp. aus-1*), which was collected from a salt lake in Western Australia. The amino-acid sequence of aR exhibits 60% homology to the sequence of bacteriorhodopsin (bR), a proton pump found in the purple membrane of *Halobacterium salinarium* [1]. In the cell membrane of *Halorubrum sp. aus-1*, aR molecules aggregate to form claret patches (claret membrane). At low ionic strength, claret membrane is separated from other membrane fractions. It was shown by atomic force microscopy that claret membrane is isolated as a sheet with a thickness of 5 nm and a diameter of 200-600 nm. The X-ray diffraction pattern from a dried film of clarlet membrane shows that aR trimers are arranged on a hexagonal lattice, whose lattice constant is only slightly larger than that observed in purple membrane. Thus, claret membrane is almost indistinguishable structurally from purple membrane.

To determine the tartial structure of aR at an atomic resolution, we have performed an X-ray crystallographic analysis using a three-dimensional crystal. In the presence of octylthioglucoside and ammonium sulfate, aR was found to crystallize into an octahedral crystal. This crystal exhibits a strong linear dichroism, suggesting that the retinal chromophore orients in perpendicular to the 4-fold axis of the crystal. Diffraction data at 3.5 Å resolution indicated that this crystal belongs to the space group P4(3)2(1)2 with cell dimensions a = b = 128.6 Å and c = 109.2 Å. A structural analysis using the molecular replacement method showed that two aR molecules are contained in the asymmetric unit. Arrangement of the retinal chromophores found in the crystal structure is consistent with the linear dichroism of the crystal It is shown that, except for the N-terminal region, the main-chain structure of aR is very similar to that of bR. All of the functionally important residues located in the protein inside have the same conformations in these two proteins. Remarkable structural differences between aR and bR are observed in the vicinity of the lipid binding site at the crevice between two monomers within the trimeric structure of bR.

P-15 SPECTRAL PROPERTIES OF 3 DIMENSIONAL BACTERIORHODOPSIN CRYSTALS

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X-Ray crystallography on 3D protein crystals provides detailed information about the structure of molecules at the atomic level. Spectroscopy, as an independent method, can give insights into the structural and functional similarities and differences between proteins in 3D crystals and in suspension or solution [1]. We have carried out a thorough analysis of absorption and fluorescence spectra of 3-D bR crystals grown in lipidic cubic phase of monoolein [2]. At room temperature spectra show differences when compared with those of PM suspensions.

a) The VIS part of the absorption spectra is broadened by 20-40nm and its maximum is blue shifted (553-560nm), while the Trp band (280 nm) is unchanged.
b) The red part of the fluorescence spectrum (max.730nm) has an excitation spectrum, which shows evidence for a certain portion of proteins being in a blue membrane like form (bR610) [3], i.e. with a protonated Asp85.
c) For excitation wavelengths between 490-530nm, a new emission band is found at 550nm, which is not present in bR-suspension. The excitation spectrum of this emission may indicate the presence of bR500 similar to bR in pink membranes [4], which is responsible for the broadening of the crystal absorption spectrum.

As we have maintained the pH value to 5.6, the appearance of bR610 and bR500 is most probably due to a change in the pK_a of Asp85. We will discuss how the crystal growth or the protein packing can bring about such modifications of the electrostatic environment.

Since crystallography, performed at LN_2 temperatures, showed a well-ordered retinal binding pocket with an all-trans retinal conformation, we are currently extending the absorption studies to low temperatures.

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P-16 EARLY EVENTS IN ACTIVE ANION TRANSPORT: HALIDE BINDING INDUCES CONFORMATIONAL CHANGES IN THE D85S MUTANT OF BACTERIORHODOPSIN

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The D85S mutant of bacteriorhodopsin uses light (energy) to pump halide and other anions across the cell membrane. We report a crystal structure for the D85S mutant of bacteriorhodopsin in which a bromide ion is bound at the same site occupied by water 402 in the wild-type protein structure. Comparison of the structure of the halide-bound state of the protein to that of the halide-free state shows that ion-binding induces significant conformational changes on the extracellular side of the protein. (1) These comparisons reveal that charge-charge interactions play an important role in lowering the Born energy associated with desolvating the halide ion both during anion transport to the binding-site and once the anion has bound to the binding-site. (2) In neither structure, however, is there a true solvent-accessible channel to the binding site, a fact that suggests that the ion-loading event requires the transient opening of a dynamic (myoglobin-like) gate. Both of these points may represent universal design principles that are used by ion pumps during their catalytic transport cycle, and that distinguish this class of molecules from ion channels.

P-17 THE OH^- ANION-PUMP MODEL IS A REASONABLE ALTERNATIVE TO THE H^+-PUMP MODEL FOR BACTERIORHODOPSIN

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It has been natural to describe wt-bR as being a light-driven proton pump. Not only does this protein generate a proton-motive force, but also the effects that selected amino acid substitutions have on pumping, and the changes in the protonation state of specific amino acid resides during the photocycle, are all explained by the H^+- pump model. On the other hand, however, all of these observations could be explained equally well by the model in which any given H^+ transfer is replaced by a corresponding OH^- transfer "in the opposite direction." High-resolution crystal structures have recently shown that water molecules exist at locations necessary to fulfill the requirements of an OH^- pump model. The presence of these strategic water molecules is not sufficient to decide between the two models, however, since the same water molecules could serve as elements of a "proton wire" pathway in the H^+- pump model. The idea that wt-bR would be an OH^- anion-pump is nevertheless a natural model to consider, since a single amino-acid substitution (D85S/T mutants) converts the protein into a halide-ion pump, as does neutralization of D85 and D212 at low pH. A new structure of the D85S mutant in the halide-bound state provides further insight into how and why it might be the case that wt-bR is an OH^- anion-pump.

P-18 COMPARISON OF TWO CRYSTAL STRUCTURES OF THE D85S (BLUE) MUTANT OF BACTERIORHODOPSIN: RELEVANCE TO THE O-INTERMEDIATE PHASE OF THE PHOTOCYCLE

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The D85S mutant of bacteriorhodopsin (bR) has been crystallized under two different environmental conditions. In both cases the space group is $C222_1$, but the in-plane c-axis differs by 10 Å for the two crystal forms. The crystal structure of the blue form of this mutant protein provides a model for the O-intermediate in the photocycle of bR in which D85 is protonated, the proton release group is deprotonated, and the retinal has reisomerized to all-trans. In the blue form the two crystal structures show similar differences in rotamer configurations of R82 and Y83 relative to the bR_{568} structure of the wild-type protein. As reported previously (Rouhani et al., JMB (2001) 313, 615-628), one O-like structure shows changes in the helix-packing for the extra-cellular ends of helices A, B, D and E. The second blue-protein structure, derived from a halide-induced purple form of the mutant protein (Facciotti et al., this meeting) does not exhibit these changes in the helix packing, however. A comparison of the "blue" crystal structures to each other and to the structure of a purple form of the mutant (or to the wild-type bR_{568} structure) suggests that there should be an intermediate (substate) between the O and bR_{568} states. The simplest interpretation of our data envisions that (1) fairly rapid changes in rotamer configuration occur in response to the deprotonation of D85 followed by (2) relaxation of helix-packing into the final bR_{568} configuration.

P-19 SUBDOMAINS IN THE F AND G HELICES OF BACTERIORHODOPSIN REGULATE THE CONFORMATIONAL TRANSITIONS OF THE REPROTONATION MECHANISM

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We have performed cysteine scanning mutagenesis of the bacteriorhodopsin mutant D85N to explore the role of individual amino-acids in the conformational transitions of the reprotonation mechanism. We have used whole-cell reflectance spectroscopy (Martinez and Turner, 2002) to evaluate the spectral properties of the 59 mutants generated during a scan of the entire F and G helices and the intervening loop region. Cys mutants were grouped into one of six phenotypes based on the spectral changes associated with their M $\leftrightarrow$ N $\leftrightarrow$ O intermediate-state transitions. Mutations that produced similar phenotypes were found to cluster in discrete molecular domains and indicate that M, N, and O possess distinct structures and that unique molecular interactions regulate the transitions between them. The distribution of these domains suggests that: 1) the extramembranous loop region is involved in the stabilization of the N and M intermediates, 2) lipid-protein interactions play a key role in the accumulation of N, and 3) the amino-acid side chain interactions in the extracellular portion of the interface between helices G and A participate in the accumulation of M.

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P-20 CONFORMATIONAL CHANGE OF THE E-F INTERHELICAL LOOP IN THE M PHOTOINTERMEDIATE OF BACTERIORHODOPSIN

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The conformation of the structured E-F interhelical loop of bacteriorhodopsin and its change in the M photointermediate were assessed by measuring the rate of reaction of sixteen single engineered cysteines along the loop with water-soluble sulfhydryl reagents. The exposure to the bulk in the unilluminated state determined with the cysteine reaction correlated well with the degree of access to water calculated from the crystallographic structure of the loop. The EF-loop should be affected by the well-known outward tilt of helix F in the M and N intermediates of the photocycle. A second mutation in each cysteine mutant, the D96N residue replacement, allowed full conversion to the M state by illumination. The reaction rates measured under these conditions indicated that buried residues tend to become more exposed, and exposed residues become more buried in M. This is to be expected from tilt of helix F. However, the observation of increased exposure of four residues near the middle of the loop, where steric effects are only from other loop residues, indicate that the conformation of the EF-loop itself is also changed. Thus, the motion of the loop in M is more complex than expected from simple tilt of helix F, and may include rotation that unwinds its twist.

P-21 ON THE ROLE OF THR90 AND PRO91 OF HELIX C: A KEY FEATURE IN THE STRUCTURE AND FUNCTION OF BACTERIORHODOPSIN

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The residues Thr90 and Pro91, located in the central part of helix C of bacteriorhodopsin (BR), are totally conserved among archaeal rhodopsins. This indicates an essential role of these residues in the structure and/or function of these proteins, and specifically in BR. Interactions of Thr90 with the peptide carbonyl oxygen of Trp86 and with the –COOH of Asp115 (helix D); van der Waals contact of Thr90 with the retinal; and kinking of helix C at the level of Pro91, may be critical for the correct location of relevant residues involved in proton transport (i.e. Asp85, Asp96), located on helix C. To analyze this aspect, we have studied point mutations of Asp115 and Pro91 to Ala, and compared them to T90A and to wild type (WT).

FTIR difference spectra obtained from films under steady-state illumination at different temperatures, present altered retinal bands in both D115A and P91A mutants, indicating an alteration in the structure of the cromophore. D115A appears more M-like under conditions supposed to yield N intermediate, a fact that coincides with T90A, but P91A is more N-like under the same conditions. Proton release and uptake monitored by pyranine absorbance changes, show a decrease of proton pumping in both mutants, but not as significant as the decrease found for T90A. Flash photolysis experiments monitored at 410 nm (M intermediate) at pH 6.5 show that both T90A and P91A have a faster M rise than WT, while the decay of M in D115A and T90A is slower than WT. The apparent pK_a of Asp85 in unphotolyzed D115A is similar to WT, whereas P91A shows a new species with maximum at 450 nm and pK_a of about 2.7.

These results suggest important roles of Thr90 and Pro91 in providing the correct location and kinking of helix C, which appear crucial for the function of BR.

P-22 EFFECTS OF THE DOUBLE MUTATION D96N/E194Q ON THE FUNCTION OF BACTERIORHODOPSIN

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Residues Asp96 and Glu194 are known as key aminoacids of the proton transport mechanism in bacteriorhodopsin (BR). To simultaneously disable the proton donor to the Schiff base and the proton release group, we have studied the double mutant D96N/E194Q of BR. It presents a number of alterations in the purple-to-blue transition, photocycle kinetics and proton pumping activity. pH titration of the mutant shows an altered Asp85 pK_a in all conditions studied (150 mM KCl, 75 mM Na_2SO_4, H_2O and deionized sample). Interestingly, a species absorbing at 440 nm appears in the deionized mutant. This red form has a pK_a of 5.6, about one pH unit higher than the Asp85 pK_a. None of the single mutants E194Q or D96N nor the WT present this red form under any of the conditions studied.

The rise and decay of the M intermediate in the double mutant D96N/E194Q was found to be pH independent, showing an M rise like E194Q and a M decay as slow as D96N. Additionally, the mutant shows an altered process of proton pumping. The absence of pyranine signal in flash photolysis experiments contrasts with the presence of proton pumping observed in reconstituted liposomes. Therefore, the absence of pyranine signal may indicate that proton release and uptake occur simultaneously.

The MN intermediate obtained by FTIR in D96N disappears in D96N/E194Q, where a N-like intermediate is trapped with a reprotonated SB like in E194Q.

Therefore, the alterations on the function of BR are stronger in the double mutant than for the sum of the single ones, suggesting a synergy between the state of protonation of both aminoacids in providing a correct environment and conformational changes for the proton transfer across the protein.

P-23 PHOTOCHEMICAL PROPERTIES OF A NEWLY ISOLATED ARCHAERHODOPSIN

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A new archaebacteria species from a salt lake in China was isolated and purified. SDS-page electrophoresis shows only one protein in the claret membrane isolated from the archaebacteria. This protein has a molecular weight close to that of bacteriorhodopsin (bR) (Li *et al.*, 2000), and has its absorption peak at around 550 nm. Its partial sequence (Wang *et al.*, 2000) shows that this bR-like protein belongs to the archaerhodopsin family, three members of which were described by Mukohata and coworkers. We tentatively named this new pigment archaerhodopsin-4 (aR4). Like bR, aR4 pumps protons from the cytoplasmic side to the extracellular side of the cell's membrane. However, no early light-induced proton release was observed at neutral pH in aR4. This suggests that in aR4 the proton release complex (PRC) in the M intermediate has a higher pK_a value than found for the M intermediate of bR (5.8). The lack of pH dependence of light-induced proton uptake between pH 6 and 8 in aR4 suggests that in the N intermediate, aR4 has a higher pK_a value for Asp96 than for bR. In bR, we assigned the pK_a of the increase of the fraction of the O intermediate at high pH (7.5) to the pK_a of Asp96 (Balashov *et al.*, 1999). In aR4, this pK_a is 8.5, consistent with Asp96 having a higher pK_a value when in the N intermediate in aR4 than in bR. In bR, we assigned the pK_a of the increase of the fraction of the O intermediate at low pH (4.3) to the pK_a of the PRC when in the O intermediate (Balashov *et al.*, 1999). In aR4, this pK_a is 5.7. These results suggest that aR4 is a light-induced proton pump as bR, but is tuned to a more alkaline environment.

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P-24 ULTRAFAST INITIAL ABSORPTION DYNAMICS OF RETINAL PROTEINS

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Initial vibrational wavepacket motions and primary reaction dynamics were studied for different Sensory Rhodopsins[1] (pSRII of Natronobacterium pharaonis and sSRI and sSRII of Halobacterium salinarum) and native and mutated Bacteriorhodopsin[2] of H. salinarum by femtosecond pump-probe spectroscopy. The laser system was operated at 1 kHz, excitation pulses were generated by a widely tunable non collinear optical parametric amplifier. Single and multi channel experiments were used to obtain time resolutions down to 30 fs over the whole visible range.

Both SRII show a similar primary reaction with a very fast (400 fs) formation of a red shifted photoproduct and the subsequencial transition to a second photoproduct (4-5 ps) with smaller red shift. This initial reaction scheme closely resembles the well known one of bacteriorhodopsin. sSRI has a much slower and biexponential decay of the initial excited electronic state (5ps and 33ps). Its reaction scheme is similar to the one of sHR[3] and the mutant sBR-D85T. (Note: These three proteins do not have a polar charge at the position corresponding to D85 in sBR). The results suggest similar underlying reaction mechanisms in the different retinal proteins and yield information for a general theoretical description.

Ultrafast measurements show a similar initial behavior in the excited electronic state. The subsequent dynamics differ: the proteins can be arranged in two groups, one with a fast subpicosecond monoexponential decay of the S1 state, the other with a biexponential and slow picosecond decay of the S1 state.

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P-25 DYNAMICS AND RETINAL STRUCTURAL CHANGES IN THE PHOTOCYCLE OF THE ARTIFICIAL BACTERIORHODOPSIN PIGMENT BR6.9

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The molecular mechanism describing the initial 200-ps of the room-temperature photocycle in the artificial bacteriorhodopsin (BR) pigment, BR6.9, is examined by both absorption and vibrational spectroscopy. The BR6.9 pigment contains a structurally modified retinal chromophore (retinal 6.9) having a six-membered carbon ring bridging the C_9=C_{10}-C_{11} bonds. Picosecond transient absorption (PTA) data show that the initial 200-ps interval contains two photocycle intermediates: J6.9 formed with a <3-ps time constant and decaying to K6.9 with a 5-ps time constant. Resonantly enhanced vibrational spectra from the light- and dark-adapted ground states of BR6.9 and the K6.9 intermediate are measured using picosecond resonance coherent anti-Stokes Raman scattering (PR/CARS) and picosecond time-resolved CARS (PTR/CARS) respectively. Each of these spectra (800-1700 cm^{-1}) contains 33 features assignable to the vibrational degrees of freedom in the retinal chromophore, and show that the retinal structures in K6.9 and in both light- and dark-adapted BR6.9 are all distinct. However, the specific mechanistic role, if any, of C_{13}=C_{14} isomerization cannot be directly identified from CARS data recorded from BR6.9 and its photocycle intermediates. Comparisons involving these PTA and CARS data from BR6.9, and analogous results obtained from the ground states and photocycle intermediates of native BR and other artificial BR pigments, demonstrate that restricting retinal motion at the C_9=C_{10}-C_{11} bonds does not generally change the initial 200-ps photocycle mechanism, but does alter the rates at which specific molecular processes occur.

P-26 A COMBINED DENSITY FUNCTIONAL AND SOLID-STATE NMR STUDY OF BACTERIORHODOPSIN STRUCTURE

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In order to relate NMR chemical shifts to molecular structure, we have conducted density functional calculations of ^{13}C and ^{15}N chemical shifts from the atomic coordinates of a number of bacteriorhodopsin related systems and compared these structure-based theoretical predictions with the experimental shifts. In establishing the accuracy of our methods, we employed two types of models compounds: uncharged extended polyene chains, which were used to investigate purely geometric effects, and retinylidene iminium salts to investigate the effect of external charges. In all the model compounds, we find good agreement between our experimental measurements and calculated shifts derived from either the B3LYP or the BPW91 density functionals (i.e., an R^2 value of ~0.95 and a root-mean-square error of ~4 ppm for the 6 compounds investigated). In the ionic systems, just one ion near the Schiff base nitrogen was necessary to obtain this accuracy. For bacteriorhodopsin itself, we considered the light adapted state and the M photocycle intermediate. The chemical shifts of the retinal chromophore were measured with care to place these all on the same shift scale, and we used x-ray diffraction generated atomic coordinates as the basis for density functional calculations. For the light adapted state, the coordinates from the 1.55 Å 1c3w Protein Data Bank structure appear to give the best agreement between theory and experiment. However none of the M state structures appear to show good agreement with experimental results. This technique appears to be a useful way to compare the qualities of different structures.

P-27 DISTORTION OF THE CHROMOPHORE OF BACTERIORHODOPSIN MEASURED VIA SOLID STATE NMR

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The role of the chromophore in the proton-motive photocycle of bacteriorhodopsin (bR) has not been fully explored. Results from a variety of spectroscopic methods qualitatively indicate twisting of the chromophore in several of the early photocycle intermediates, but X-ray structures of various photocycle intermediates do not provide a consistent picture of the chromophore conformation. Using solid-state NMR techniques, we have previously found deviations from planarity in the H-C14-C15-H torsion angle that increase in the first half of the photocycle. We have now extended our measurements to the H-C15-N-H torsion angle and again find significant distortion from a relaxed, planar configuration already in the light adapted state. These distortions suggest that the chromophore is in an activated or pre-loaded state prior to photoisomerization and that significant further evolution of the chromophore conformation occurs after photoisomerization event.

P-28 INTERNUCLEAR DISTANCE MEASUREMENTS IN UNIFORMLY $^{13}C,^{15}N$ LABELED PEPTIDES AND PROTEINS

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Magic-angle spinning solid-state NMR (SSNMR) determinations of internuclear distances (e.g., between ^{13}C-^{13}C and ^{13}C-^{15}N) have relied heavily on the use of isolated spin-1/2 pairs. Studies of multiply $^{13}C,^{15}N$ labeled systems have the potential for greater efficiency. However, in these systems, accurate measurements of long-range dipolar interactions are complicated by the presence of stronger couplings. We have recently described an experiment, frequency selective REDOR (FSR) [1], which circumvents some of the problems associated with multiple spin systems. Here we present applications to accurate measurements of weak ^{13}C-^{15}N dipolar couplings in two [U-$^{13}C,^{15}N$]-labeled molecules. In the tripeptide, N-formyl-Met-Leu-Phe, 16 distances were measured between 2.5 and 6 Å with a precision of ~ 0.1–0.3 Å. The distance constraints were subsequently used in the calculation of the three-dimensional structure of the tripeptide. In bacteriorhodopsin, two distances were measured across the active site: in the light-adapted state, the distances from the Schiff base nitrogen to the carboxyl carbons of the aspartate sidechains were 4.7±0.3 Å for D85 and 4.9±0.5 Å for D212.

[1] Jaroniec, C. P.; Tounge, B. A.; Herzfeld, J.; Griffin, R. G. *J. Am. Chem. Soc.* **2001**, *123*, 3507-3519.

P-29 DYNAMIC NUCLEAR POLARIZATION OF BACTERIORHODOPSIN

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It is generally accepted that solid state NMR offers unique probes of membrane structure and dynamics. For example, both distances and torsion angles have been measured to a high degree of precision (typically ±0.3Å and ±5-10°, respectively) in the resting state and photointermediates of bacteriorhodopsin (bR) and rhodopsin. However, because of low sensitivity, NMR spectra presently require extended periods (~10 days) of signal averaging. In this poster we describe an approach – high frequency dynamic nuclear polarization (DNP) — that we have recently developed in our laboratory that could significantly alter this situation.

In DNP, the large polarization of unpaired electrons is transferred to nuclei, significantly enhancing the nuclear magnetic resonance (NMR) signals. Paramagnetic centers can be introduced into the sample by addition of the stable nitroxide free radical TEMPO to a solution containing a membrane protein. The polarization transfer is accomplished by irradiating the center of the EPR spectrum of the sample with microwaves. To generate the microwaves for the irradiation we have developed a series of high frequency microwave oscillators (gyrotrons) that provide 10-100 watts of power in the 100-500 GHz frequency range. These millimeter wave frequencies correspond to the EPR frequencies of g~2 electrons at ^{1}H NMR frequencies of 200-750 MHz where solid state NMR is currently performed.

To date we have implemented DNP/NMR experiments at ^{1}H frequencies of 211 MHz and 380 MHz, using gyrotrons operating at 140 and 250 GHz. In the experiments described here, bR samples were dispersed and frozen in water/glycerol solutions containing TEMPO. At 90 K, the ^{13}C and ^{15}N signals of the protein were enhanced by factors of 50-60, which translates to a reduction of $>10^3$ in signal acquisition time. Thus, experiments that have required several days can now be completed in a few hours and with higher accuracy. Examples of structural studies that now become feasible will be described in detail.

P-30 PROGRESS TOWARDS COMPLETE CHEMICAL SHIFT ASSIGNMENTS FOR UNIFORMLY LABELED BACTERIORHODOPSIN IN PURPLE MEMBRANE

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Recent studies have demonstrated the feasibility of resolving and assigning chemical shifts in moderately large solid-state proteins. Of particular interest is the application of these methods to determine the structures of membrane proteins, most of which cannot be crystallized readily. In order to address some of the issues involved, we have obtained high resolution solid state NMR spectra of uniformly ^{13}C and ^{15}N labeled bacteriorhodpsin at field strengths of 700 and 750 MHz. After carefully optimizing sample conditions, the 1D ^{13}C and ^{15}N spectra displayed significant resolution. In addition, N-Ca, N-Co, N-Ca-Cb and C-C experimental data sets have been recorded. These 2D spectra have already yielded partial chemical shift assignments and the prospects for full chemical shift assignment in the native protein are discussed.

P-31 THE L INTERMEDIATE OF BACTERIORHODOPSIN PHOTOCYCLE IS STABILIZED BY CYTOPLASMIC DOMAIN WATER

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During the K to L transition, the Schiff base nitrogen forms a stronger H bond. Several water molecules also undergo perturbations which result in stronger H-bonding and increase in intensity of their O-H stretch vibration bands. At least two of these water molecules with particularly intense bands are localized to the cytoplasmic domain of the pigment. These waters affect deprotonation of the Schiff base in the L to M transition. Mutation of cytoplasmic residues, Thr46, Asp96 and Val49 depleted, restored, and intensified the water bands, in parallel with acceleration, restoration and deceleration of the L to M transition (reviewed in [1]).

In this study we identified the HOOPS bands of the Schiff base in L and found that they are coupled to water vibrations (exhibit a shift in ^{18}O water). Mutations of the residues mentioned above affected the Schiff base N-H HOOPS bands in a similar way to their effects on the water bands. We conclude that the Schiff base in L interacts with water molecules in the cytoplasmic domain [2].

The role of cytoplasmic water in the formation of L was further underlined by unusual features of the L93M and W182F mutants. We found that these mutations (but not mutations of Phe219) facilitated L formation so that L was observed at very low temperature, 80K. In the WT L is formed only at > 140 K. Moreover, in the initial (unphotolyzed) states of these mutants, water molecules exhibit a hydrogen bonding pattern and band intensity which in the WT they acquire only in L. These results indicate that the formation of L involves rearrangement of residues and water molecules in the region surrounded by Leu93, Trp182 and the retinal. We propose that water molecule(s) enter cavities in the Schiff base-13-methyl group region formed by the photoisomerization, and participate in the stabilization of the L state.

[1] Maeda, A. *Biochemistry (Moscow)*, 66, No11, Special Issue on Bacteriorhodopsin and Other Retinal Proteins, 1256-1268.
[2] Maeda, A. et al. (2002) Biochemistry, 41, 3803-3809.

P-32 DYNAMICS OF INTERNAL WATER MOLECULES IN BACTERIORHODOPSIN

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Internal water molecules play an important rule in the proton conduction process in Bacteriorhodopsin (bR). Since high resolved X-ray structures have become available locations of such water molecules under deep-temperature and crystal conditons are known. Detailed information of the dynamics of these waters at room temperature and non crystalline circumstances has not been available yet. To provide such information and thus gaining a dynamical model of the bR ground state and the mechanism of proton transfer, we set up a simulation of bR over several ns covering the protein in its complete quartenary structure and explicit membrane / water environment. Based on recent X-ray data (Belrhali et al. 1999) the bR-trimer was inserted in a fully solvated 16 by 16 POPC-bilayer patch (Tielemann et al. 1999) resulting in a final system size of approximately 84000 atoms. Molecular dynamics calculations of 5 ns of unrestrained MD were performed using the GROMACS package and force field (van der Spoel et al. 1999). Besides a structural and energetic analysis, special emphasis has been laid on the study of internal water molecules' mobility, rendered by spatial density distributions and a detailed analysis of dynamic forming of H-bonds.

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Tieleman, Sansom, Berendsen (1999). Biophys. J. *76*, pp. 40-49

van der Spoel D., van Buuren A.R., Apol E., Meulenhoff P.J., Tielemann D.P., Sijbers A.L.T.M., Hess B., Feenstra K.A., Lindahl E., van Drunen R., Berendsen H.J.C. (1999)

P-33 THE ORIGIN OF THE CONTINUUM BANDS OF BACTERIORHODOPSIN REVEALED WITH STEP SCAN FTIR SPECTROSKOPY IN COMBINATION WITH SITE DIRECTED MUTAGENESIS

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Bacteriorhodopsin has been extensively studied with FTIR in the region 1800 cm^{-1} to 900 cm^{-1}, where its amino acids and the retinal show well defined bands (1).

With time-resolved step scan FTIR difference spectroscopy (2), a small but broad continuum band is observed between 1900 cm^{-1} and 1800 cm^{-1}, which is caused by a delocalised proton within a protonated H-bonded network on the proton release side (3). This $H_5O_2^+$ complex is located in a pocket between Arg 82, Glu 204 and Glu 194 close to water 404 near the extracellular surface (4).

Now the spectral range is extended up to 3000 cm^{-1}:

The absorbance change between 2500 cm^{-1} and 2100 cm^{-1} is shown to be caused by heating of the sample due to the actinic laser flash (5), in contrast to an early interpretation by Wang and El-Sayed (6). Besides this heating artefact another continuum band is observed between 3000 cm^{-1} and 2500 cm^{-1}. The molecular origin of this continuum band will be discussed.

(1) Gerwert, K., *Biol. Chem.* (1999) 380, 931-935

(2) Rammelsberg, R., Heßling, B., Chorongiewski, H., Gerwert, K., *Applied Spectroscopy* (1997) 51, 558-562

(3) Rammelsberg, R., Huhn, G., Lübben, M., Gerwert, K., *Biochemistry* (1998) 37, 5001-5009

(4) Spassov, V. Z., Luecke, H., Gerwert, K., Bashford, D., *J.Mol Biol* (2001) 312, 203-219

(5) Garczarek, F., Wang, J., Gerwert, K., El-Sayed, M., (in preparation)

(6) Wang, J., and El-Sayed, M., *Biophysical Journal* (2001) 80, 961-971

P-34 PEROXYNITRITE STRESS AND MEMBRANE PROTEIN ALTERATIONS

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Besides its interest as a light-driven proton-pump, in previous years bacteriorhodopsin (BR) successfully serves as a model system to study the specific properties of integral membrane proteins. We have employed bacteriorhodopsin to evaluate the validity of "The free radical hypothesis of ageing" and "The membrane theory of ageing", using the peroxynitrite anion ($ONOO^-$) as reactive species. This oxidizing species is involved in pathophysiology and cellular defense against infection.

Treatment of the membrane protein bacteriorhodopsin in the purple membrane or the water soluble protein bovine serum albumin (BSA) with peroxynitrite (PN) leads to the formation of 3-nitrotyrosine (NT). As in the case of BSA there are no other modifications of amino acids detectable in BR besides NT. There is no reaction of PN with retinal according to UV-VIS-spectroscopy. The concentration of $ONOO^-$ needed to induce alterations are strikingly higher for PM as compared to BSA. In the case of PM just 0,2 % of the applied ONOO- (in absence of CO_2) react to NT (BSA: 5 %). This is most likely the consequence of the special arrangement of BR in PM. The presence of CO_2 enhances nitration of tyrosines (at higher CO_2-concentrations this effect is diminished). The rate constants of the formation and the decay of the M-intermediate are more or less halved by treatment of PM with PN in the absence of CO_2. With increasing concentrations of CO_2, the values of the rate constants approximate to those of untreated PM.

Besides investigation of trimeric BR in the PM, containing only few saturated isoprenoid lipids, experiments were performed with BR reconstituted in different lipid environments. Under the conditions chosen BR was present as mobile monomer, completely surrounded by the lipids DOPC (18:1) or DLPC (18:2). Due to lipid peroxidation the damage of BR in BR-DLPC-liposomes is augmenting until complete destruction of the chromophore and loss of protein integrity.

P-35 SUB-SECOND PROTON-HOLE PROPAGATION IN BACTERIORHODOPSIN

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The dynamics of proton transfer between Purple Membrane and bulk have been recently investigated by the Laser Induced Proton Pulse Method (LIPPM). Following a delta function release of protons to the bulk, the system was noticed to regain its state of equilibrium within few hundreds microseconds. These measurements set the time frame for the relaxation of any state of acid-base disequilibrium between the bacteriorhodopsin's surface and the bulk. It was also deduced that the released protons react with the various proton-binding sites within less than 10 μs (Checover, Marantz, Nachliel, Gutman, Pfeiffer, Tittor, Oesterhelt, and Dencher (2001) Biochemistry **36** 13919-13928).

In the present study, we monitored the photocycle and the proton cycle of photo excited BR, in the absence of added buffer, and calculated the proton balance between the Schiff base and the bulk phase in a time-resolved mode. It was noticed that the late phase of the M decay (beyond 1 ms) is characterized by a slow (sub-second) relaxation of disequilibrium, where more protons are found in the bulk than the deprotonated Schiff base can accept. Thus, it appears that the internal proton donor (D96) is compensated by bulk proton donors at a rate slower than the proton transfer to the deprotonated Schiff base, generating a 'proton-hole' in the cytoplasmic section of the protein. The velocity of the hole propagation is modulated by the ionic strength of the solution and by selective replacements of charged residues on the interhelical loops of the protein at domains that seem to be remote from the intra-protein proton conduction trajectory.

P-36 TWO PROCESSES LEAD TO STABLE ALL-*TRANS* AND 13-*CIS* ISOMERS EQUILIBRIUM IN DARK-ADAPTED BACTERIORHODOPSIN; EFFECT OF HIGH PRESSURE ON BACTERIORHODOPSIN, BACTERIORHODOPSIN MUTANT D96N AND FLUORO-BACTERIORHODOPSIN ANALOGUES

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Bacteriorhodopsin (bR), a retinal protein found in the purple membrane of the bacterium *Halobacterium salinarum,* exists in two distinct forms: light-adapted and dark-adapted. Light-adapted bR contains an all-*trans* chromophore. Dark-adapted bR is a mixture of all-*trans* and 13-*cis* isomers. The question what processes lead to stable equilibrium of cis-trans isomers in dark adapted bR is still open.

The combination of absorption spectroscopy and extraction techniques was applied to study the effect of high pressure on dark-adapted state of bR, 14-(12-, 10-)Fluoro-bR, D96N bR mutant, and 14-(12-,10-)Fluoro-D96N. The evidences are presented that at high pressure, the isomers' equilibrium is shifted from all-*trans* isomers towards the 13-*cis* isomers. Two groups of values of calculated molar volume changes indicate that there are at least two different processes leading to stable all-*trans* and 13-*cis* isomers equilibrium called the dark-adapted bacteriorhodopsin. The first process may be attributed to changes in the distances and rearrangement of functionally-important residues and retinal Schiff base. It is suggested that the moved residues (probably Asp-212 with the contribution of Tyr-185 and/or Asp-85) closer to the chromophore could catalyse its trans-cis isomerization. These changes require smaller pressure changes and induce larger volume changes (large-volume-change process). The second process may be attributed to the formation of the three hydrogen bonds that additionally decrease the volume and strengthen further stabilisation of the 13-*cis* isomer. To induce these changes, larger changes of pressure are required and the final molar volume changes are smaller (small-volume-change process). The total molar volume change between bacteriorhodopsin all-*trans* and bacteriorhodopsin 13-*cis* in dark-adapted state of native bacteriorhodopsin was found to be about – 30 ml/mol.

P-37 A FLASH-PHOTOLYSIS STUDY OF RETINAL ANALOGUES INCORPORATED IN BACTERIO-OPSIN

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All-*trans*-retinal is the chromphore of the transmembrane protein bacteriorhodopsin (bR). As a result of the initial photoisomerization, the chromophore undergoes a reaction sequence, involving structural and conformational changes, which is well researched.

It was of interest to study whether retinal analogues show a similar photocylce in the same membrane apoprotein in order to gain a better understanding of the chromophore and its interaction with the protein environment.

The analogues used in this study[1] feature five membered heterocycles (thiophene, furan and pyrrole) instead of the cyclohexene ring in retinal. They also differ in steric demands from the natural bR-chromophore, by the introduction of methyl groups at different positions of the heterocycles and by changing the linkage of the side-chain to the heterocyclic ring.

Seven all-*trans* forms of the analogues were successfully assembled into the membrane apoprotein which was produced from the chromophore deficient mutant *Halobacterium salinarium JW 5*, and the reaction was observed by UV/Vis-spectroscopy.

To find out whether the assembled analogues behave similar to the wild type protein the samples were studied by extensive flash-photolysis experiments in the range from 350 to 650 nm. The results were analysed by a multiexponential global fit method. The goal of the experiments is to determine the absorption maxima of the different intermediate states and their lifetimes.

The results of these experiments are presented, as well as a preliminary interpretation.

[1]Synthesized by Michael Dornbusch as part of his doctoral thesis (Dissertation, Universität Düsseldorf, 2001)

P-38 VOLUME AND ENTHALPY CHANGES OF PROTON TRANSFERS IN THE BACTERIORHODOPSIN PHOTOCYCLE

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The volume and enthalpy changes associated with the proton translocation steps during the bacteriorhodopsin (BR) photocycle were studied on purple membrane (PM) preparations using 20 microsecond to second time resolved photo-pressure measurements. The enthalpy data show only moderate positive enthalpy changes thus making unlikely inherent entropy-driven steps in the BR photocycle. The volume data show two increases followed by a decrease with time constants of 50 us, 1 ms and 6 ms at pH 7, 10 mM phosphate buffer, 100 mM KCl, 25 C, with the slowest time increasing with increasing pH. The fast expansion, the slow expansion and the last volume contraction correspond to the L→M, M→N and the N→O→BR transitions, respectively, of the optical cycle. These indicate the proton release, proton translocation within the BR and the proton uptake steps. The volume change differences between phosphate and Tris buffers in the first and third steps are smaller than expected and not in the same ratio. Either the quantum yield of proton pumping is lower than the photocycle yield or, more likely, the binding of buffer ions to the PM modifies their volume and enthalpy changes on protonation. The volume change data can be interpreted by the electrostriction effect as charge is cancelled and formed during the proton transfers. A conformational change with large positive volume is then required in the 1 ms (M→N) step and its reversal in the 6 ms (N→O→BR) step.

Buffer	ΔV0	ΔV1	ΔV2	ΔV3	ΣΔV	ΔH0	ΔH1	ΔH2	ΔH3	ΣΔH
Phos 7.0	-1.1 (0.3)	+14.0 (0.2)	+3.6 (0.2)	-16.5 (0.3)	0.0 (1.0)	-204 (5)	-6 (2)	+1 (3)	+12 (5)	-197 (15)
Phos 8.0	-1.3 (0.3)	+13.6 (0.1)	+8.0 (0.2)	-19.8 (0.3)	0.5 (0.9)	-193 (5)	-10 (2)	-39 (3)	+24 (5)	-217 (14)
Tris 8.4	-0.2 (0.3)	+6.3 (0.1)	+8.9 (0.4)	-15.6 (0.5)	-0.6 (1.3)	-170 (5)	-5 (1)	-13 (5)	-21 (6)	-210 (16)

ΔV in A^3 and ΔH in kJ, both per absorbed photon of 205 kJ. (n) rms error.

P-39 OPTIMIZATION OF BACTERIORHODOPSIN-BASED THIN FILMS FOR USE IN A VARIETY OF BIOELECTRONIC DEVICES

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Optimizing the orientation of purple membrane patches deposited onto conductive substrates is crucial to the development of bacteriorhodopsin (bR) -based bioelectronic devices. BR-based field-effect transistors (FET), artificial retinas, photovoltaic films and electronically erasable spatial light modulators all require that the protein be oriented and strongly coupled to the substrate in order to maximize photovoltaic response. Over the past two decades, electrophoretic sedimentation and electrostatic layer by layer assembly of proteins and polymers have become reliable techniques for attaching oriented organic materials onto semiconductive surfaces. By using these techniques, and by controlling the atmospheric humidity and pH of the deposition solution, excellent photovoltaic response of bR films is obtained. In addition, atomic force microscopy (AFM) combined with techniques in non-contact electrostatic force microscopy are now being used to monitor and detect the photovoltaic signals generated from thin films of bR exposed to a variety of environmental conditions. We will report on the current status of these studies along with the methods of preparing optimized photovoltaic thin films of bacteriorhodopsin.

P-40 THE BRANCHED PHOTOCYCLE INTERMEDIATES P AND Q IN BACTERIORHODOPSIN

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The bacteriorhodopsin branched-photocycle intermediates **P** and **Q** are studied with respect to chromophore isomeric content, photochemical origin, kinetic heterogeneity and photoreversibility. These blue-shifted species are compared to products with similar spectroscopic properties generated via thermal denaturation. We observe that the thermal and photochemical species differ in both isomeric content and protein environment. Sequential two-photon activation of glycerol suspensions of bacteriorhodopsin containing low concentrations of water (<15% V/V water/glycerol) form high yields of **P** state. Relatively large photo-stationary state populations of both intermediates can be generated in both 85% V/V glycerol suspensions and polyacrylamide gels. At ambient temperature, both intermediates can be fully converted back to **bR** with blue light. Chromophore extraction and HPLC analysis reveal that **P**, **Q**, and the spectrally similar thermal products contain predominantly the 9-*cis* retinal chromophore. Time-resolved spectroscopy reveals that the **P** state is actually comprised of two components with maximum absorptivities at 445 and 525 nm. The molar absorptivities of the chromophore band maxima of the **P** and **Q** states in an 85% V/V glycerol/water suspension at pH 7 are $\varepsilon(\mathbf{P}_{445})$= 47,000 M^{-1} cm^{-1}, $\varepsilon(\mathbf{P}_{525})$= 39,000 M^{-1} cm^{-1}, and $\varepsilon(\mathbf{Q})$= 33,000 M^{-1} cm^{-1}.

P-41 MUTAGENESIS OF RESIDUES AFFECTING THE O STATE IN BACTERIORHODOPSIN

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The nature of the **O** state in the bacteriorhodopsin photocycle is not well understood. This state is the final intermediate in the main photocycle, and is the most red-shifted of the intermediates. It cannot be trapped at low temperatures, despite that fact that it is the longest-lived intermediate. From a device standpoint, the **O** state is important as it represents the origin of the branching photochemistry that generates the **P** and **Q** states. The latter states are used for long term data storage in both volumetric and holographic data storage systems. Site-directed and semi-random mutagenesis techniques have been used to alter the lifetime and yield of the **O** state. Mutants that affect the **O** state are generally placed into two categories: those that inhibit chromophore isomerization from 13-*cis* to all-*trans*, and those that slow down deprotonation of Asp-85. Some site-directed mutants have been found that extend the **O** state lifetime (normally 6-8 milliseconds in wild-type) to as much as 1 second. Our current research focuses on using semi-random and random mutagenesis coupled with high-throughput screening to discover promising **O**-state mutants. Semi-random mutagenesis has already uncovered a triple mutant that provides the highest yield of **O** state observed so far and appears to involve a mechanism different from the two outlined above.

P-42 ANISOTROPIC PHOTOELECTRIC RESPONSE IN ORIENTED BACTERIORHODOPSIN FILMS

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We present experimental results on the anisotropic photoelectric response of dried oriented bacteriorhodopsin (bR) films. Both photo-excitation and photochemical bleaching processes were used to introduce photoelectric anisotropy in the BR films. Polarization-dependent photovoltages were measured across the ITO/bR/ITO/glass structures. The influences of the pump and bleach light intensity on the anisotropic effect were investigated. Polarization sensitive BR-photodetectors are proposed, based on these features. We will also discuss in this presentation a theoretical model to explain the observed anisotropic effects and compare the calculated results with the experimental data.

P-43 THE BRANCHED PHOTOCYCLE OF BACTERIORHODOPSIN

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The branched photocycle of bacteriorhodopsin (BR) begins with the photochemical formation of the P-state from the transient O-state intermediate. Optimization of this sequential two-photon process depends in part on increasing the O-state population, enhancing the O to P phototransition, and eliminating the side reactions that might form the P-state via thermal or direct photochemistry. In order to study the branched photocycle, we measure the kinetics of the O-state using a single photon process and a sequential two-photon process respectively. Our results show that the time constant of the O-state for a single photon process is always larger than that of a sequential two-photon process. To maximize the O-state population and kinetics we study the effects of temperature, pH, and salt concentration. The rate of formation of the P- and Q-state increases by an order of magnitude for every 8°C up to approximately 42°C. The optimal temperature is found by an Arrhenius plot to be 40°C on our apparatus. Increasing pH reduces the amount of O-state, but increases the rate of formation of the P- and Q-state by an order of magnitude for every 2 pH units. Increasing the ionic strength does not change the amount of O-state, but decreases the rate of formation of the P- and Q-state with a linear correlation.

P-44 PHOTOELECTRIC PROPERTIES OF BACTERIORHODOPSIN: A KELVIN FORCE PROBE MICROSCOPY STUDY

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The purple membrane of *Halobacterium salinarum* was investigated by Kelvin Force probe Microscopy (KFM), a powerful tool that can be used to investigate the local electric potential distribution of samples with nanometer-scale lateral resolution. Purple membrane consists of bacteriorhodopsin (BR) and lipids in approximately a three to one ratio (BR to lipid). Membrane patches prepared at pH 7 or 10 were immobilized on mica, dried, and photoexcited at 635 nm in order to generate a photostationary state between the bR and M intermediates. Both the wild-type and the D96N mutant were examined. Images were recorded in both normal AFM mode and a modified KFM mode, under both dark and illuminated conditions. A photovoltaic effect was observed upon photoexcitation, which was more prevalent at higher pH. The observed response is presumably due to light-induced charge separation in bacteriorhodopsin. Further studies are needed to verify the results and to draw more concrete correlations with the bacteriorhodopsin photocycle.

P-45 EVALUATION OF PURPLE MEMBRANE PATCH SIZE AND BACTERIORHODOPSIN PHOTOCYCLE KINETICS AS A FUNCTION OF HIGH-FREQUENCY SONICATION

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Atomic Force Microscopy (AFM) was used to examine the effect of sonication on the average size of purple membrane patches isolated from *Halobacterium salinarum*. Buffered purple membrane solutions were sonicated for intervals up to one minute at different sonication amplitudes. The converter and 1/8" microtip sonication probe were tuned to vibrate at 20 kHz. The resulting purple membrane (PM) patches were dried on freshly cleaved mica immediately after sonication to immobilize for imaging. The average pre-sonication patch size was measured to be approximately 800 μm. A linear correlation between the average patch diameter and sonication duration was demonstrated: after 60 seconds of continuous sonication (96 μm tip vibrational amplitude) the average PM patch size was reduced to 137 nm. After 60 seconds at an amplitude of 48 μm an average diameter of 577 nm was observed. Kinetics studies were also performed to determine any changes in the photocycle. Sonication of aqueous samples and immediate observation of the bR-, M-, and O-states indicated a possible effect on the photocycle due to sonication.

P-46

ELECTRIC-FIELD EFFECTS IN DRY FILMS OF WILD-TYPE BACTERIORHODOPSIN AND ITS MUTANTS

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The application of an external electric field (up to ~7 10^5 V/cm) to dry oriented films of wild-type bacteriorhodopsin (BR) produces the red-shifted product whose optical properties are similar to those of the acid-blue form of the protein. This process, probably due to field-induced Asp-85 protonation, is temperature dependent with E_a~36 kJ/mol (at relative humidity 12%). The electroabsorption changes are completely vectorial and can be obtained only if field direction is opposite to that of proton pumping (field-I).

The laser-induced amplitude of the absorbance changes at 410 nm (M-form) has a linear dependence on the external electric field. The amount of M increases when the field is directed as for proton pumping (field-II) and decreases with a change of the field polarity. The rise time of the M-form is slightly sensitive to the external field in both directions while its decay is retarded drastically under field-II. The time constants of the three kinetic components of decay process (~100 ms, ~0.6 s and ~5 s) remains unaltered but relative amplitude of the slowest component increases greatly.

In some BR mutants with low proton affinity for the Schiff base (D85N, D85N/D96N, D85T/E204Q) the application of an external electric field causes the deprotonation of the Schiff base with M formation in the dark. Based on the temperature dependence of this effect, which is seen in oriented films in the field-II direction only, an activation energy ~68 kJ/mol is calculated.

Comparison of the results with a simple model suggests that the external field may change the pK_a of the Schiff base or Asp-85 by 1-2 units.

P-47 DYNAMIC HOLOGRAPHY RECORDING FOR STUDY OF PHOTOINDUCED PROCESSES IN THE BACTERIORHODOPSIN AND ITS 14-FLUORO DERIVATIVES

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The first results on the dynamic holography recording on the 14-fluoro bacteriorhodopsin (BR) derivative are presented. A study of 14-F BR derivatives is of particular interest since this pigment demonstrates the red shift of the initial absorbance [1]. This permits utilization of inexpensive semiconducter lasers as a part of the optoelectronic device. A holographic kinetics on the 14-F wild type (WT) BR is compared with one on the WT BR. We used cw He-Ne laser, λ=633 nm, for dynamic holography recording. The cw He-Cd laser, λ=442 nm, was used for the activation of the short wavelength-absorbing M-intermediates. The self-diffraction holographic recording kinetics on the WT BR consists of two stages: an initial peak followed by a relaxation to a steady-state [2]. The additional blue light illumination by He-Cd laser causes the steady-state value to increase leaving the initial peak value unchanged. This observation can be interpreted in the framework of the irreversible step between the "fast" intermediate M_1 and "slow" intermediate M_2 in the WT BR [3]. In contrast to this, an initial peak is not observed on the 14-F WT BR without additional blue light illumination. The He-Cd laser causes the initial peak on the 14-F WT BR to arise and grow. The steady-state value is unchanged. This suggests that molecules accumulate in the early M_1 intermediate in the 14-F WT BR. Thus, the separation of the individual species of M intermediates can be easily reached by the dynamic holographic recording.

Acknowledgement. This work was in part supported by grant UB2-2427-KV-02 from the CRDF (to E.K and N.B.) and grant 01-04-48198 from the RFBR (to A.D.).

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P-48 EFFECT OF RELATIVE HUMIDITY ON PERFORMANCE OF 14-FLUORINE BACTERIORHODOPSIN GELATIN FILMS

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Recently a study was performed to investigate the photoinduced transformation of 14-fluorine (F) bacteriorhodopsin (BR) gelatin films based on both wild type (WT) and the D96N mutant in comparison with those for water suspensions of the same pigments [1]. The main result of this study of the films is: there is no formation of a red shifted species at 660 nm for both types of gelatin films as opposed to the observations of a red shift in the same pigments in water suspensions. In addition, there are no fundamental differences in the photoinduced behavior between WT and D96N as observed with the water suspensions [1]. In this poster we outline the possible explanations of these experimental results. Basically they arise from the kinetic characteristics for gelatin films which are different from those for water suspensions. The main factor in altering the kinetics is a relative humidity of gelatin samples. So the next step for this study was to vary the humidity of the gelatin samples to find the optimal value to obtain the desired properties (large red shift) of 14-F BR gelatin films. Dehydration effects on gelatin films of the 14-F WT and 14-F D96N were studied. Spectral and kinetic characteristics were monitored over a wide range of relative humidity (18% - 92%). Such a range of humidity has been defined for each pigment where spectral and kinetic characteristics were changed in a desired direction. The results obtained confirm our earlier suggestion about the existence of two photoinduced processes which occur in suspension and films of the corresponding pigments [1,2].

Acknowledgement. This work was supported by the RFBR grant № 01-04-48198.

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P-49 CIRCULAR DICHROISM STUDY OF BACTERIORHODOPSIN VARIANTS; WHAT IS THE ORIGIN OF THE BIPHASIC CD BAND?

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Although bacteriorhodopsin (bR) is one of the best characterized membrane proteins, the nature of its biphasic CD spectrum is not completely evident. Is it caused by *excitonic* interaction between the chromophores in the trimers or by the protein heterogeneity[1]? To get stronger arguments for or against the excitonic interaction we considered a few approaches. This presentation is comprised of three parts.

First, to support the evidence for exciton coupling the heterochromophoric approach was explored based on the stepwise limited regeneration of apo-protein with native retinal, followed by recombination with synthetic retinal analog. Investigation of the heterochromic samples based on various combinations (2:1, 1:2, 1:1:0) demonstrated the additive character of CD spectra.

The second part of CD study represents data for various bR mutants. Supported by cross-linking to confirm the identity of trimeric organization, these results demonstrate that although native bR and the mutants possess the same aggregation, they may have drastically different CD spectra.

The third part represents CD study of apo-protein partially regenerated with retinal so that only one per 3 binding sites is (randomly) occupied by chromophore. CD behavior of the bR subunit still immobilized in the membrane but not involved into trimeric aggregation was investigated in presence of sucrose and other polyhydric alcohols. The data confirm that the shape of CD spectrum is extremely sensitive to microenvironment: a variety of CD shapes – from the single positive band to various bilobes and to the single negative one – could be observed for the same sample depending on the percentage of sucrose. These results support the evidence that the biphasic CD could be explained as a superposition of CD spectra of variously distorted bR subunits in the trimers of the purple membrane.

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P-50

BACTERIORHODOPSIN BLUE MUTANTS: CHEMICAL ENHANCEMENT

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Blue species of bacteriorhodopsin are very attractive for optical applications due to the red-shifted absorption spectra and a possibility to explore the advantages of branch photocycle [1, 2]. However, blue mutants are known to share low quantum efficiency of the *all-trans* -to- *9-cis* chromophore conversion [3]. Efforts to achieve a higher sensitivity for blue mutants led to a number of D85X and D85N/XX-double mutants [4].

In this presentation two approaches to alter photochromic properties of blue mutants are considered: a) chemical enhancement by pH tuning and b) enzymatic modification. The data are compared for D85N, D85N/D96N and D85N/V49A blue mutants investigated in the form of suspension and as dry gelatin-based films.

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P-51 PROTEIN-BASED INTEGRATED OPTICAL SWITCHING AND MODULATION

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Coupling of optical data-processing devices with microelectronics, as well as with sensory functions, is one of the biggest challenges in molecular electronics. Intensive research is going on to seek suitable nonlinear optical materials, that could meet the demanding requirements of optoelectronic applications, especially regarding high sensitivity and stability. In addition to inorganic or organic crystals, biological molecules have also been considered for use in computer hardware among which the bacterial chromoprotein, bacteriorhodopsin (bR) generated the most interest. bR undergoes enormous absorption – and concomitant refractive index – shift upon initiation of a cyclic series of photoreactions by a burst of actinic light. This effect can be exploited to create highly versatile all-optical logical elements. We demonstrate the potential of this approach by investigating the static and dynamic response of optical waveguides coated with a thin layer of bacteriorhodopsin, working as an active nonlinear optical material to produce a variety of integrated optical switching and modulation effects.

P-52 THE LASER-INDUCED BLUE STATE OF BACTERIORHODOPSIN: MECHANISTIC AND COLOR-REGULATORY ROLES OF METAL IONS AND PROTEIN-PROTEIN AND PROTEIN-LIPID INTERACTIONS

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In this poster we characterize the roles of the purple membrane (PM) lattice, the I^*_{460} bacteriorhodopsin (BR) photocycle intermediate, and divalent cations in the conversion of PM to laser-induced blue membrane (LIBM; λ_{max} = 605 nm) upon irradiation with 532 nm laser pulses by contrasting the photoconversion of PM with that of monomeric BR solubilized in reduced Triton X-100 detergent. Monomeric BR forms a recently reported (see Masthay, *et. al.*, *J. Am. Chem. Soc.* **2002**, *124*, 3418-3430) *colorless monomer* photoproduct which manifests an unstructured band near 360 nm similar to the 360 nm band in LIBM. The exceptionally large two-photon absorptivities ($\geq 10^6$ cm^4 sec $molecule^{-1}$ $photon^{-1}$) of both of these photoconversions, the temporal and spectral characteristics of pulses which generate LIBM, and the PM→LIBM action spectrum all indicate that the generation of both LIBM and colorless monomer is mediated by a sequential biphotonic mechanism in which I^*_{460} absorbs the second photon, leading to the reduction of the Schiff base terminus of the retinyl chromophore and – in the case of PM – subsequent conformational perturbations of the PM lattice which induce the removal of Ca^{2+} and Mg^{2+} ions from the PM surface. The mechanistic similarity of the PM→LIBM and monomer→colorless monomer photoconversions indicates that the photochemistry is not mediated by exciton interactions between proximal retinyl chromophores.

P-53 ISOSBESTIC REGIONS INDUCED IN THE ABSORPTION SPECTRUM OF BACTERIORHODOPSIN BY PHOTOINDUCED CHANGES IN LIGHT SCATTERING

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An isosbestic region forms on the long-wavelength edge of the λ_{max} = 555 nm chromophore band of monomeric bacteriorhodopsin (MonBR = purple membrane solubilized in Triton X-100 detergent) upon irradiating aqueous suspensions of MonBR with intense 532 nm laser pulses. The total absorbance of MonBR is equal to the sum of a genuine Beer's law absorbance component originating from the retinyl chromophore of BR plus a light-scattering component originating from the micelles. Hence it is possible that the isosbestic region near 620 nm may originate from laser-induced decreases in chromophore absorbance occurring concomitantly with laser-induced increases in light scattering from the micelles. In this poster we demonstrate that isosbestic regions similar to those observed in MonBR can be generated by superimposing the spectra of mixtures of varying concentrations of bromocresol purple dye (λ_{max} = 590 nm; used to model the BR chromophore absorbance) and milk (used to mimic light scattering by micelles). In addition, we present a theoretical model assuming a Gaussian chromophore absorption profile and a λ^{-n} scattering profile which also reproduces the MonBR isosbestic region. Our results suggest that the isosbestic region observed during the generation of MonBR may arise without the formation of a true bathoproduct. While bromocresol purple dye specifically models the chromophore absorption of BR, our theoretical model is completely general, suggesting that such light scattering-induced isosbestic regions may be common in the spectra of turbid suspensions of chromophoric proteins, hence warranting caution before automatically ascribing such isosbestic regions to genuine bathoproducts.

P-54 THE EFFECTS OF PROTIC AND NONPROTIC ACIDS ON THE SPECTRA OF CAROTENOIDS

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Both protic and nonprotic acids induce large (~10,000 cm^{-1}) bathochromic shifts in the absorption spectra of carotenoids, resulting in characteristic orange-to-blue color changes. In spite of their similarity to the bathochromic "opsin" shifts observed upon protonating retinyl polyenes, the chemical origins of the carotenoid bathochromic shifts are not well-understood, nor are there any reports which systematically explore the effects of different acids on the absorption spectra of carotenoids. The carotenoid bathochromic shifts may originate from two possible sources: (i) protonation-induced stabilization of the optically-absorbing, "ionic" $^1B_u^+$ excited state with respect to the "covalent" $^1A_g^-$ ground state, as is observed with retinyl polyenes, and/or (ii) acid-induced formation of carotenoid radicals which have bathochromically-shifted absorption spectra with respect to the parent carotenoid. Potential source (i) is applies only to protic acids, whereas potential source (ii) may apply to both protic and nonprotic acids. In this presentation we report our recent characterization of the effects of solvent polarity, free radical quenchers, and Brønsted bases on the bathochromic shifts induced in the spectra of β-carotene by a series of protic and nonprotic acids. We rationalize our findings in terms of potential sources (i) and (ii), and describe potential new studies which may further elucidate the similarities and differences between the acid-induced bathrochromic shifts in carotenoids and retinyl polyenes.

P-55 PHOTODEGRADATION RATES OF BACTERIORHODOPSIN

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The rates of photodegradation of bacteriorhodopsin were measured over the wavelength range of 450 to 650 nm using a 10 hz pulsed laser light source operated between 4 and 15 mW/cm2. The action spectra show significant photodegradation rates between 480 and 540nm, which corresponds to the strong visible absorption band of bacteriorhodopsin. The kinetic data implies a two-step consecutive first-order reaction mechanism. The first step is the photoconversion of bacteriorhodopsin to the previously reported "blue state" that is very stable(>24 hours). The second step is the photoconversion of the "blue state" to an irreversible product, that was previously noted by the three UVpeaks at 340, 360, and 380 nm. It is proposed that the final product is a form of anhydrovitamin A.

P-56 THE AZIDE-INDUCED BACTERIORHODOPSIN-LIKE PHOTOCYCLE OF SALINARUM HALORHODOPSIN

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Salinarum halorhodopsin (sHR), a small protein in the cell membrane of Halobacterium salinarum, is a light driven chloride ion pump. The photocycle of sHR was investigated in the presence of azide. The azide binds to the halorhodopsin with 150 mM binding constant, when no chloride is present in the solution, and with 1 M binding constant in the presence of chloride. It is demonstrated that the azide-binding site is different from that of chloride, the chloride only influences the binding constant. The analysis of the absorption kinetic signals proves the existence of two parallel photocycles:

$K \Leftrightarrow M_1 \Rightarrow M_2 \Leftrightarrow sHR' \Rightarrow sHR$ and

$K^{cis} \Rightarrow sHR$

The spectra of intermediates were determined with time-resolved spectroscopy. Intermediates L, N and O were not detected, which could originate from kinetic reason. The multiexponential rise and decay of the M intermediate could be explained by the introduction of the "spectrally silent" intermediates M_1, M_2 and HR', HR respectively. The electric signal measurements revealed the existence of a positive component, which appears during the M_1 to M_2 transition, which is probable, the accessibility switch step of the photocycle. The absorption kinetic signal measured during continuous illumination of the sample proved that the azide photocycle of the salinarum halorhodopsin is not a side reaction of the proton transporting photocycle, as was considered earlier.

P-57 OCCUPANCY OF TWO PRIMARY CHLORIDE-BINDING SITES IN THE HALORODOPSIN OF *N. pharaonis* AS A CONDITION FOR ACTIVE ANION TRANSPORT

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Flash-induced voltage response of halorhodopsin *N. pharaonis* in presence of Cl anions reveals two main components. Fast component with $\tau \sim 1$ μs has amplitude near 4% of total signal and slow one with $\tau \sim 1$ ms takes the rest. Both time constants did not demonstrate valuable Cl concentration dependences. Apparent K_D of voltage response equals 6 mM with Hill's coefficient 1.0 [1]. At the same time spectral changes (absorbance maximum shift) displays K_D=1 mM with Hill's coefficient 0.75 [2]. Spectral changes was attributed to chloride anion binding near the Schiff base. The non-unit Hill's coefficient and discrepancy between apparent K_D of spectral changes and voltage response was non-elucidated.

In present work the set of 15 visible spectra of halorodopsin *N. pharaonis* at chloride concentration from 100 μM to 100 mM (500 mM Na_2SO_4, 10 mM MES, pH=6.4) was measured. The SVD analysis of spectra reveals 2 chloride-dependent components. Bell-shaped form of second Cl-dependent component unambiguously demonstrates existence of two chloride-binding sites. The global fitting of SVD components found K_D's 0.47 mM and 5.2 mM with unit Hill's coefficient. The second K_D coincides with apparent K_D of photovoltage response. The main spectral shift is associated with K_D = 0.47 mM. It can be interpreted in terms that anion binding site close to Schiff base has lower K_D, but without filling second anion-binding site no anion transport activity occur. The binding site with chloride affinity 5.2 mM can be attributed to surface one. Small spectral influence of surface binding site can be explained by electrostatic interaction between surface and Schiff base located anions. Surface position of second binding site allows to explain absence of chloride dependency in the main photovoltage time constant [1].

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P-58 ANION-BINDING TO THE BLUE FORM OF HALORHODOPSIN FROM *NATRONOBACTERIUM PHARAONIS*

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Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) is a powerful method for studiing infrared biological materials in general, and biological membranes in particular. It allows the investigation of the structure of proteins in intact biological membranes in an aqueous environment.

This method has been used to analyze the conformational changes of the membrane protein halorhodopsin from *Natronobacterium pharaonis* (pHR), a light-driven chloride pump, during anion binding to the blue form of HR which is obtained by removing all intrinsic anions (undergoing a spectral shift from 578 nm to 600 nm). A thin multilayer film of blue HR was prepared on a diamond internal reflection element. Overlaying this dry film with anion free water stabilized the blue form and made it possible to add a defined anion concentration (chloride, bromide, jodide, nitrate and azide) to the buffer medium during the experiment.

The resulting difference-spectra between the blue and the purple form of pHR revealed characteristic vibrational bands. The largest can be ascribed to the chromophore and amide I changes. The latter represent structural changes of the protein during anion uptake.

Comparing the difference spectra induced by chloride *vs.* nitrate shows interesting differences and similarities which help to understand the anion binding mechanism of this anion pump. In order to identify bands caused by bound nitrate isotopically labelled (^{15}N) spectra have also been obtained.

The spectra are compared to those of the photocycle´s O intermediate which also putatively represents an anion-free form of HR.

Acknowledgement
We thank A.A. Wegener for providing pHR expressed in E. Coli.
This work was supported by the Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie (to F.S.).

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P-59 EFFECT OF S130A MUTATION OF *pharaonis* HALORHODOPSIN ON THE CHLORIDE BINDING AND THEP HOTOCYCLE

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Bacteriorhodopsin (bR) and halorhodopsin (hR), which exist in the membrane of *Halobacterium salinarum*, are light-driven ion pumps. In spite of high similarity of primary and tertiary structures between bR and hR, these membrane proteins transport different ions, proton and chloride, in the opposite direction. From alignment of the amino acid sequences, Thr-89 of bR is homologous to Ser-115 of hR from *Halobacterium salinarum* (*s*hR). X-ray structure of *s*hR has revealed that OH group of this residue directly interacts with Cl^-. Thus, Ser-115 of *s*hR is expected to play an important role in the Cl^- binding and the transport. In this study, we expressed the wild type hR from *Natronobacterium pharaonis* (*p*hR) and its S130A mutant in *Escherichia coli* in order to clarify the role of Ser-130 on the Cl^--binding affinity and the photocycle reaction. Here, Ser-130 of *p*hR corresponds to Ser-115 of *s*hR. The Cl^--binding induces the blue-shift of the absorption maximum, λmax. From the titration with Cl^- on λmax, the binding affinity of S130A became quite lower than that of WT (WT 6 mM, S130A 89 mM). Furthermore, from the flash photolysis with pulse laser (532 nm), the reaction rate of S130A from the O intermediate to the hR ground state was found to become apparently slower than that of WT. The singular value decomposition (SVD) and the global fitting analyses of the photocycles were performed to identify all photointermediates and determine the reaction rates. For the mutant, the branching pathway from L intermediate directly to the ground state of *p*hR was observed. The present results indicate that the Ser-130, which contacts with Cl^- in the retinal pocket, is one of the essential residues for the chloride binding and transport mechanism.

P-60 ASP76 IS THE PRIMARY PROTON DONOR IN THE TWO-PHOTON SIGNALING PATHWAY OF SENSORY-RHODOPSIN-I

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Sensory Rhodopsin-I (SRI), an archaeal color discriminating phototaxis receptor, occurs in the cell membrane complexed with its transducer protein HTR1. In natural light SRI exists as a mixture of two spectral forms, SRI_{587} and S_{373} in photochromic equilibrium. The S_{373} form, generated by excitation of SRI_{587} by orange light (one-photon process), either decays thermally in a few seconds into SRI_{587} or rapidly photoconverts back into SRI_{587} in ca. 200 ms upon excitation with UV-blue light (two-photon process). These reactions generate attractant and repellent phototaxis responses respectively. The two photon reaction in HTR1-free SRI or HTR1-complexed SRI involve intermediates absorbing at 410nm (S^b_{410}), 510nm (S^b_{510}), and 550nm (S^b_{550}). In wild-type strains photoexcitation of S_{373} generates in <50ns a S^b_{410}/S^b_{510} mixture in thermal equilibrium. The $S^b_{410} \rightarrow S^b_{510}$ conversion involves protonation of the Schiff-base, presumably from a protein donor group. In HTR1-free SRI-D76N the $S^b_{410} \rightarrow SRI_{587}$ reaction takes several seconds to complete and bypases the S^b_{510} form, indicating that D76 is the native proton donor. In D76N the observed slow reprotonation must occur via an alternate protein donor or from the aqueous medium via an as yet unidentified path.

Supported by a UCSC faculty research grant.

P-61 INFLUENCE OF ARG72 OF *PHARAONIS* PHOBORHODOPSIN ON THE M-INTERMEDIATE DECAY AND THE PROTON PUMPING ACTIVITY

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Pharaonis phoborhodopsin (*p*pR, also called pharaonis sensory rhodopsin II, NpSRII) is a photophobic receptor of *Natronobacterium pharaonis*. X-ray structures of *p*pR show the different direction of the side chain of Arg72 from that of the corresponding residue (Arg82) of bacteriorhodopsin, BR. For BR, this residue is considered to play an important role in the proton pumping. In order to investigate the role of Arg72 in *p*pR, we constructed Arg72 mutants of R72A, R72K and R72Q using an E. coli expression system, and measured the photocycle and proton pumping activities. The pH-titration curves on the absorption maximum of the mutants were shifted to alkaline in comparison of that of the wild-type. This may imply the increase of pKa of D75, suggesting the presence of the (probably electric) interaction between D75 and Arg72. Rate constants of the M-decay of the mutants were 3-7 times faster than that of the wild-type, and the periods for the completion of the photocycling were also reduced. Using SnO_2 electrode, the rate of the photo-induced proton transport was measured using inside-out membrane vesicles from *E. coli*. The photo-induced proton pumping activities were estimated from the initial pH changes after the corrections that are 1) the percentages of the D75-associated form (which has no pumping activity) and 2) the photocycling rates. R72A and R72Q showed the reduced activity while R72K did not reduce the activity.

We previously proposed that in the M-decay, protons are transferred to the deprotonated Schiff base through both the cytoplasmic (CP) and extracellular channel (EC). This is the reason for the weak proton pumping activity of *p*pR, in addition to the slow photocycling rate. The present observations indicate that the proton transfer through EC is obstructed partially by the positive charge of guanidium of Arg72.

P-62 PROTON UPTAKE AND RELEASE DURING THE PHOTOCYCLE OF RECONSTITUTED *PHARAONIS* PHOBORHODOPSIN (SENSORY RHODOPSIN II) REVEALED BY THE PHOTOELECTROCHEMICAL CELL

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Pharaonis phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, psRII) is a receptor for the negative phototaxis of *Natronobacterium pharaonis*. The amino acid sequence and the crystal structure of *p*pR are homologous to those of bacteriorhodopsin (bR), a well known light-driven proton pump. It has already been reported that *p*pR also pumps protons during its photocycle [1] while its activity is weaker than bR, and that the proton uptake and release of solubilized *p*pR coincide respectively with the formation and decay of O-intermediate [2].

In this study, photoelectrochemical responses of *p*pR reconstituted into phospholipids were measured using SnO_2 glass transparent electrode which is sensitive to the pH change. In contrast to the solubilized state, fast proton release which coincided with the formation of M-intermediate was observed during the photocycle of reconstituted *p*pR under neutral to acidic pH conditions. Introduction of mutation at Asp193 which locates near the extracellular surface and corresponds to Glu204 of bR eliminated the fast proton release. This result may indicate the participation of Asp193 to the so-called proton releasing group which produces the fast proton release in the reconstituted *p*pR. It was also shown that the extent of fast proton release of the wild-type was affected in a Cl^- concentration-dependent manner suggesting the binding of Cl^- around the proton releasing group. Molecular mechanism for the proton release of *p*pR will be discussed by comparing with that of bR.

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P-63 TYR-199 AND CHARGED RESIDUES OF *PHARAONIS* PHOBORHODOPSIN ARE IMPORTANT FOR THE INTERACTION WITH ITS TRANSDUCER

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Pharaonis phoborhodopsin (*p*pR; also *p*sRII) is a retinal protein in *Natronobacterium pharaonis* and is a receptor of negative phototaxis. It forms a complex with its transducer, *p*HtrII, in membranes and transmits light signals by protein-protein interaction.

Luecke *et al.* [1] and Royant *et al.* [2] solved the X-ray crystallographic structure of *p*pR and proposed the hypothesis for the binding site of *p*pR to *p*HtrII. Luecke *et al.* proposed that Tyr-199 on face I of *p*pR is a key residue. Royant *et al.* proposed the importance of a charged surface patch on the cytoplasmic side of *p*pR.

Tyr-199 is conserved completely in phoborhodopsins among a variety of archaea, but it is replaced by Val (for bacteriorhodopsin) and Phe (for sensory rhodopsin I). Previously, we (revised to *J. Photochem. Photobiol.*) showed that analysis of flash-photolysis data of a complex between D75N and the truncated *p*HtrII (t-Htr) gives a good estimate of the dissociation constant K_D in the dark. To investigate the importance of Tyr-199, K_D of double mutants of D75N/Y199F or D75N/Y199V with t-Htr was estimated by flash-photolysis and was about 10-fold larger than that of D75N, showing the significant contribution of Tyr-199 to binding. The K_D of D75N/t-Htr complex increased with decreasing pH, and the data fitted well with the Henderson-Hasselbach equation with a single pKa of 3.86 $\pm$ 0.02. This suggests that certain deprotonated carboxyls at the surface of the transducer (possibly Asp-102, Asp-104 and Asp-106) are needed for the binding.

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P-64 EXTRACELLULAR CHANNEL OF PHOBORHODOPSIN (SENSORY RHODOPSIN II) EXHIBITS DECREASED PROTON CONDUCTIVITY AS COMPARED TO BACTERIORHODOPSIN

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The photocycle of *pharaonis* phoborhodopsin (ppR) is much slower than that of bacteriorhodopsin (bR). This is due primarily to the very slow O → ppR transition, which involves deprotonation of the primary proton acceptor Asp75. This slow step might be caused by decreased proton conductivity of the extracellular channel in ppR compared to that in bR. In order to check this we conducted steady state and time resolved titrations of Asp75 in ppR. Protonation of Asp75 in ppR is accompanied by a red shift of the absorption band. It occurs with pK_a 3.4 (major) and 5.2 (minor). The second transition might be caused by an influence of the protonation of some residue in the extracellular channel (possibly Asp193) on the proton affinity of Asp75. Stopped flow experiments provided information on the rate of protonation and deprotonation of Asp75 in the initial state. Upon increasing the pH from 2 to 7 deprotonation of Asp75 in 150 mM KCl occurs with τ 60 ms. This is > 20 fold slower than in bR (< 3 ms) but comparable with the rate obtained for the T205V mutant of bR which greatly decreases the rate of Asp85 deprotonation (from <3 ca. 40 ms). This result is consistent with the structural data indicating that extracellular domain is more hydrophobic in ppR than in bR and the residue analogous to Thr205 in bR is replaced by Val in ppR. The rate constant of Asp75 deprotonation is pH independent but exhibits a weak Cl dependency: it accelerates three fold upon increasing the [Cl^-] from 0 to 2 M by replacing 1 M Na_2SO_4 with 2 M NaCl. The O → ppR transition is still much slower than the deprotonation of Asp75 in unphotolyzed ppR. The decay of the O intermediate is biphasic. At 20 °C the time constant of fast component of O decay is ca. 1 s. At 40 °C O decays with time constants 170 ms (80%) and 3.3 s (20%). The much slower decay of O compared to the deprotonation of Asp75 in the initial state indicates that ppR undergoes light-induced structural changes which strongly decrease the rate of Asp75 deprotonation in O thus extending the lifetime of the signaling state in ppR.

P-65 COLOR DETERMINNG FACTORS IN *PHARAONIS* PHOBORHODOPSIN BY THE ANALYSIS OF CHIMERIC PROTEIN WITH BACTERIORHODOPSIN

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Pharaonis phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sRII) is a photophobic sensor in *Natronobacterium pharaonis*. *p*pR has a shorter absorption maximum (λmax, 500 nm) than those of other archaeal rhodopsins such as bacteriorhodopsin (BR; 570 nm). Recently, the crystallographic structure of *p*pR was reported. The structure around chromophore in *p*pR is similar to that in BR. However, the hydrogen bond of protonated Schiff base (PSB) in *p*pR is stronger than that in BR from FT-IR study [1]. We previously reported from FT-IR study that the hydrogen bond of PSB in *p*pR multiple mutant having the retinal binding site residues of BR (BR/*p*pR) remained strong [2]. These facts may indicate that the environment around PSB in *p*pR slightly differs from that in BR, and there exist some long-range interactions determining the structural features around PSB.

Here, we constructed chimera proteins between BR and *p*pR to investigate the long-range interactions. First, we constructed chimera proteins separating at loop CD (B-ABC/P-DEFG and P-ABC/B-DEFG), where B-ABC/P-DEFG means the chimera composing of (helices A, B and C of BR) and (helices D, E, F and G of *p*pR). Unfortunately, B-ABC/P-DEFG is not expressed in *E. coli*. On the other hand, the λmax of P-ABC/B-DEFG was 544 nm similar to that of BR expressed in *E. coli* (λmax, 549 nm). These results indicate that the major factor(s) determining the difference of λmax between BR and *p*pR exist in helix DEFG. At the present time, we are trying to specify the sites which give rise to the color tuning between BR and *p*pR.

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P-66 TIME-RESOLVED STEP-SCAN FTIR STUDIES OF THE PHOTOREACTION OF SENSORY RHODOPSIN II FROM *NATRONOBACTERIUM PHARAONIS*

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The sensory rhodopsin II pigments mediate photophobic response to blue light. In addition, some proton pumping activity has been reported recently, especially in the presence of azide (*1*). However, in order to mediate the photophobic response, it interacts with its membrane-bound transducer via helix-helix interaction, which is modulated in the signaling state of the receptor thought to be the state with deprotonated Schiff base and protonated counterion D75. In order to obtain a deeper insight into the molecular events involved in receptor activation, we studied the photoreaction with time-resolved step-scan FTIR spectroscopy addressing these different issues. The investigations show that the photoreaction is characterized by K, L, M_1, M_2 and O intermediates in agreement with time-resolved UV-*vis* studies (*2*). The two M states differ only in the protein conformation and in both the counterion D75 is protonated. In O, the chromophore is back-isomerized to all-*trans*, and an additional carboxyl group becomes protonated. In the presence of azide, M decays fast to a red-shifted intermediate, in which the chromophore is still-13-*cis* and the slow backisomerization and protein conformational changes determine the almost unchanged cycling time. We hypothesize that the large increase in proton pumping observed under azide is due to a two-photon process, the second photon being absorbed by this red-shifted intermediate, thereby shortening the photocycle. The photocycle of the D75N mutant, in which the deprotonation of the Schiff base is inhibited, is considerably accelerated and characterized by red-shifted intermediates which differ slightly in protein and chromophore structure among each other. The protein conformational changes, however, are very different from those observed in the M states. In addition, also the early red-shifted intermediate is different from the K state, both with respect to chromophore and protein structure. Finally, we studied the photocycle in the presence of the minimal transducer (*3*) and could identify characteristic changes in the amide-I spectral range for the signaling M state.

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P-67 GEL CHROMATOGRAPHIC CHARACTERIZATION OF *p*SRII/*p*HTRII COMPLEX

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The *Natronobacterium pharaonois* sensory rhodopsin II (*p*SRII) and its transducer protein (*p*HTRII) interact and cooperate with each other in order to mediate phototaxis responces. *p*HTRII is also known as an analog of eubacterial methyl-accepting chemotaxis proteins and forms homo dimer when functionally expressed in the halobacterial membrane. On the other hand, although *p*SRII has many common structural characters to bacteriorhodopsin, that does not form trimer even in its crystal unit cells.

*p*SRII can be expressed abundantly in the *E. coli* membrane as photoactive form. Solubilized and partially purified recombinant protein is very stable and is able to store in the cold and dark place at least for a few years. Some "aged" samples' retention volume of gel filtration chromatography was shown that the molecular mass of the protein was smaller than just solubilized one from *E. coli* membrane. The estimated molecular weights were about 28k and 54k, respectively. It is possible that the aged sample seemed be degradated but the same aged sample has none of significant changes were observed its spectrum and photoactivity characteristics, and after re-treatment of ion-exchanged column, that had an identical retention volume to fresh one. This result suggests that the*p* SRII monomers are able to form homo dimer.

Unlike *p*SRII, *p*HTRII cannot be expressed in *E. coli* membrane but its truncated membrane associated fragment, namely, tHtr. This fragment is assumed forming dimer in a similar manner to the intact *p*HTRII and the estimated molecular mass from gel filtration supports this surmise. In addition of purified tHtr to *p*SRII solution, the retention volume of the photoactive peak was shifted. Such a modulation is not contradicted by a former study about availability of interaction between tHtr and *p*SRII even in the presence of detergent. These results indicate that the biologically active unit of photosignaling seems to be a hetero-tetramer comprised of *p*HTRII dimer and *p*SRII dimer.

P-68 A SINGLE-RESIDUE SWITCH IN NATURAL VARIANTS OF MARINE PROTEORHODOPSINS

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By flash photolysis of the pigments expressed in *E. coli*, we show that green-absorbing proteorhodopsin (G-PR; λ_{max} 527 nm; identified in ocean surface plankton) and blue-absorbing proteorhodopsin (B-PR; λ_{max} 490 nm; identified in 75-meter deep ocean plankton and in Antarctica) differ in their photochemistry. In *E. coli*, whole cells G-PR undergoes a faster (~5.5-ms) photocycle with M and O accumulation and B-PR undergoes an order of magnitude slower (~60-ms) photocycle with only slight M accumulation. By site-specific mutagenesis of residues in the predicted retinal-binding pockets of both pigments, we found that a single residue substitution (L105Q in G-PR versus Q105L in B-PR) nearly completely interconverts the two pigments. L105Q G-PR is blue-shifted, gains fine-structure, and its photocycle becomes as slow as that of B-PR without pronounced M accumulation. Q105L B-PR is green-shifted, loses fine-structure, and gains accumulation of M. G-PR has been previously shown to carry out efficient electrogenic light-driven proton ejection from *E. coli* cells and has been also shown to exhibit the same photocycle kinetics in sea surface plankton. We show here that both G-PR and B-PR outwardly pump protons from right-side-out *E. coli* vesicles. Mutagenesis, proton flux, and photocycle measurements confirm that the proton-acceptors and donors to the Schiff base during the pumping process in each pigment are Asp97 and Glu108, respectively, as shown previously for G-PR. However, in energized *E. coli* cells at pH 7, photoexcitation of B-PR causes a net proton influx, unlike G-PR that drives net proton efflux. We explain this different behavior as caused by the less efficient proton ejection of B-PR that generates proton currents dominated by a triggered proton influx through voltage or pmf-gated channels in the *E. coli* cell membrane. These measurements and calculations of light availability at the depth of B-PR raise the question of whether the physiological function of B-PR is regulatory (i.e. it may function as a sensory rhodopsin) rather than photoenergizing as is G-PR.

P-69 PROTON TRANSFERS IN THE PHOTOCHEMICAL REACTION CYCLE OF PROTEORHODOPSIN

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We determined the spectral and photochemical properties of proteorhodopsin (PR) [1] in order to compare its proton transport steps to those of bacteriorhodopsin (BR). Static and time-resolved measurements on wild-type PR and several mutants were done in the visible and infrared (FTIR and FT-Raman). Assignment of the observed C=O stretch bands indicated that Asp-97 and Glu-108 serve as the proton acceptor and donor, respectively, to the retinal Schiff base, as do the residues at corresponding positions in BR, but there are numerous spectral and kinetic differences between the two proteins. There is no detectable dark-adaptation in PR, and the chromophore contains nearly entirely all-*trans* retinal. Because the pK_a of Asp-97 is relatively high (7.1), the proton-transporting photocycle is produced only at alkaline pH. It contains at least seven transient states with decay times in the range from 10 μs to 200 ms, but the analysis reveals only three distinct spectral forms. The first is a red-shifted K-like state. Proton release does not occur during the very slow (several milliseconds) rise of the second, M-like, intermediate, consistent with lack of the residues facilitating extracellular proton release in BR. Proton uptake from the bulk, presumably on the cytoplasmic side, takes place prior to release ($\tau \sim 2$ ms), and coincident with reprotonation of the retinal Schiff base. The intermediate produced by this process contains 13-*cis* retinal as does the N state of BR, but its absorption maximum is red-shifted relative to PR (like the O state of BR). The decay of this N-like state is coupled to reisomerization of the retinal to all-*trans*, and produces a state that is O-like in its C-C stretch bands, but has an absorption maximum apparently close to that of unphotolyzed PR.

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P-70 TIME-RESOLVED FT-IR SPECTROSCOPY OF PROTEORHODOPSIN

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Proteorhodopsin (pR) is a bacteriorhodopsin homolog, recently discovered in oceanic bacteriorplankton, which functions as a light-driven proton pump. Its photocycle has initially been characterized by laser flash-induced absorption spectroscopy and a positive differential absorbance at 410 nm in the absorption spectra is believed to be indicative of the presence of the M intermediates. We have measured the time-resolved FT-IR difference spectra of pR intermediates reconstituted in DMPC vesicles at pH 9.5. A peak at 1757 cm^{-1}, characteristic of the M state, appears with a rise time of 60 μs. This is a bit slower than the rise times for M formation and H^+ - release that were reported earlier [Krebs et. al., *BMC Physiology*, 2:5, 2002].

P-71 A HIGHLY-PERTURBED ARG82-VIBRATION IN M: CONFIRMATION BY TIME-RESOLVED FT-IR SPECTROSCOPY OF BACTERIORHODOPSIN CONTAINING ^{15}N-LABELED ARGININES

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Arginine-82 (R82) of bacteriorhodopsin (bR) has been recognized as an important residue due to both its conservation among the archaeal rhodopsins and mutation of it results in conformational changes and loss of fast proton release. NMR studies showed that R82 experienced a highly asymmetric environment during the M state [Petkova et al., *Biochemistry* 38, 1562-1572, 1999]. Previous FT-IR studies of wide-type bR, the R82A mutant, and the R82C mutant containing an isotopically-modified "pseudoarginine" side chain covalently linked to the cysteine indicated that a peak near 1550 cm^{-1} was likely assigned to a highly-perturbed C-N stretch vibration of R82 [Hutson et al., *Biochemistry* 39, 13189-13200, 2000]. We have now measured the time-resolved FT-IR difference spectra of bR intermediates in the wild-type protein, containing either natural-abundance isotopes (^{14}N-Arg-bR) or with all 7 arginines selectively and uniformly labeled with ^{15}N at the 2 η-nitrogens (^{15}N-Arg-bR). Comparisons of the spectra from the two proteins show that the 1556 cm^{-1} band due to M in ^{14}N-Arg-bR reproducibly loses substantial intensity in ^{15}N-Arg-bR. We conclude that a strong C-N stretch vibration of arg-82 can indeed be assigned to a highly-perturbed frequency near 1555cm^{-1} in the M state of wild-type bR.

P-72 ELECTROSTATIC COUPLING OF PROTEORHODOPSIN CHROMOPHORE TO ITS ASPARTATE COUNTERION AND NEARBY CONSERVED ARGININE: SIMILARITIES AND DIFFERENCES WITH BACTERIORHODOPSIN

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Like its homolog, bacteriorhodopsin (bR), proteorhodopsin (pR) is thought to function as a light activated proton pump. Titration of the retinylidene chromophore's aspartate counterion (Asp97 in pR) shifts the visible absorption spectrum. In wild-type pR, this "red-to-purple" transition occurs with a pK_a of 8.0, over 5 units higher than the purple-to-blue transition in bR. In contrast to D85N bR, the D97N mutant of pR has a λ_{max} of 547 nm, identical to the wild type at pH 7; furthermore this λ_{max} does not shift over the pH range 5-10.5. Surprisingly, when Arg94 is mutated to a neutral residue (cysteine), a blue-shifted λ_{max} of 525 nm is seen at at pH 7. This R94C mutation does not have a substantial effect on the pK_a of Asp97 titration; nor does subsequent treatment of the R94C mutant with guanidinium compounds. This is in contrast to bR where the R82C mutation elevates the pK_a of Asp85 by ~5 units, and chemical reconstitution with guanidinium compounds can restore this pK_a almost back to the value of 2.5 seen in the wild type. The shift in the pK_a of Asp97 to 8.0 in pR from its value of 2.5 in bR is thus explained as being likely due to weaker coupling, i.e. greater physical separation, between Arg94 and Asp97 in pR, in comparison to Arg82 and Asp85 in bR. In combination with previously published results, these data indicate that fast light-driven H^+ release from the pR/bR family of proton pumps is not dependent on any tightly-conserved initial configuration of the residues involved in the H^+ release group.

P-73 RHODOPSIN ACTIVATION IN NONANIMAL EUKARYOTES

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In addition to the ubiquitous presence of rhodopsins in animal photoreceptors, rhodopsins are also found in at least three major non-animal eukaryotic Kingdoms. We have studied them in *Chlamydomonas reinhardtii*, a green alga plant, in *Allomyces,* a chytridiomycete fungus and in *Peranema trichophorum,* a Heteronematales euglenoid. *Peranema* is a colorless flagellate whose probability of changing direction depends on light as it does in *Halobacterium.* Unlike *Chamydomonas* and *Allomyces*, *Peranema*'s do not track the light and hence are not phototactic in the accepted sense. Comparison of amino acid sequences (three in fungi and two in algae) indicates a common ancestry with prokaryotic and animal rhodopsins.

Study of these organisms has provided insight into the evolution of animal rhodopsins. As far as is known they do not pump protons. Consequently, a long isomerizable chromophore is apparently not essential. In each organism n-hexenal forms as functional a photoreceptor chromophore as does retinal in spite of its small size and inconsequential change in shape if it isomerizes. Further, nonisomerizable analogs such as those formed from naphthalenes and many ring-locked analogs work well in *Chlamydomonas.* This implies that without significant structural change of the chromophore, the structural changes in the opsin are sufficient to result in *in vivo* behavioral changes. Potentially similar structural changes have been observed in bacteriorhodopsin with nonisomerizable chromophores (Aharoni et al., 2001). The suggested importance of isomerization for animal rhodopsin function, implies that during evolution the studied animal photoreceptors have added dependencies on isomerization and the photocycle that were less important in ancestral forms. Presumably this came about to reduce the noise in systems having many orders of magnitude more receptor pigment molecules.

REFERENCE:

Aharoni, A. et al. Biochemistry (Moscow) 66:1210-1219 (2001).

P-74 MCCE CALCULATIONS SHOW HOW SMALL STRUCTUAL DIFFERENCES STABILIZE PROTON TRANSFER IN BACTERIORHODOPSIN

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In the reaction cycle of bacteriorhodopsin, each step of proton transfer changes the protein charge distribution. There are only small structural differences between the intermediates in crystal structures of the protein trapped in bR, early M and late M states, with an RMSD of ≈1Å. MCCE calculations, which equilibrate side chain ionization states and positions and buried water occupancies, were carried out for these structures. The calculations show that each structure supports the experimentally observed proton distribution. In each there is a net charge of –1 on the Schiff base, Asp85 and 212. The negative charge is stabilized by the backbone positive potential and the buried ionized Arg82. One cluster proton changes its location, moving from the Schiff base finally to Asp85 (Fig.). As the proton moves within the central cluster, there are protonation changes at the proton release group. A large pK_a shift leads to proton release in the late M state. The pH dependence of the ionization states of each residue and accompanying conformational changes of polar side chains, water molecules and the changes of pair-wise electrostatic interactions stabilizing the different ionization states will be shown in detail. Supported by NSF-MCB-9629047.

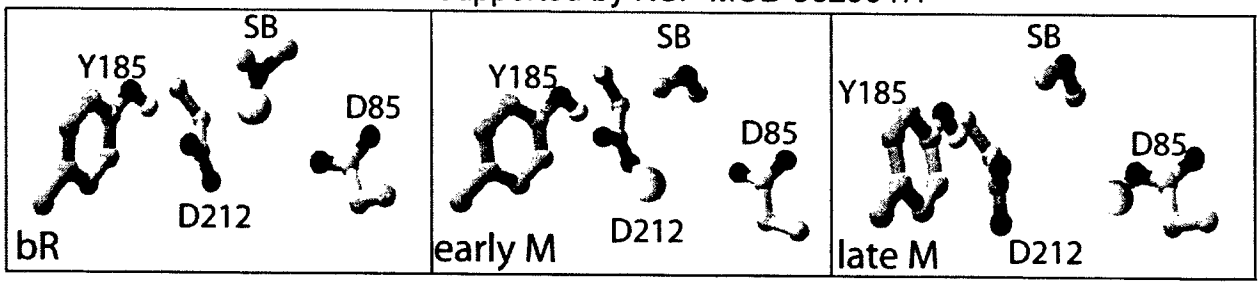

Figure. In the middle of the protein, one proton (shown as larger ball) moves between the Schiff base, Asp85 and 212 in the bR, early M and late M states. In the bR state, favorable interactions of the Schiff base with the two acids support the ionization of all 3 sites. In the M states, the Schiff base faces away from the two acids, reducing each favorable interaction by ≈3 kcal/mol. The Schiff base is now deprotonated. In early M, Asp85 is 60% and 212 40% protonated (shown). In late M, there is a movement of helix F moving Tyr185 towards Asp212, favoring its ionization. Asp85 is now fully protonated.

P-75 ELECTROSTATIC CALCULATIONS OF IONIZATION STATES AND PROTON TRANSFER IN THE BACTERIORHODOPSIN PHOTOCYCLE

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We have carried out calculations of the protonation states of key residues in the proton transfer pathway of bacteriorhodopsin in various states of the photocycle (BR, K, L, M, O) whose structures have recently been solved experimentally. We use a continuum solvation model coupled with an explicit lipid membrane representation. Our results are in general agreement with the accepted sequence of protonation/deprotonation events observed experimentally during the photocycle, such as proton release from an extracellular site and movement of the proton from the Schiff base to ASP-85. We also find that the the protonation state of ASP-96 depends strongly on its local environment, and we have modeled some aspects of the structural rearrangements which may occur in the protein in the M->N step of the photocycle to facilitate re-protonation of the Schiff base from ASP-96.

P-76 COMPUTER SIMULATIONS OF PROTON TRANSPORT PATHWAYS IN BACTERIORHODOPSIN

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The first proton transport step in the bacteriorhodopsin photocycle is the transfer of the Schiff base proton to the Asp85 amino acid. To understand the mechanism of this process the transition state structures need to be isolated and characterised. We have combined the quantum mechanical/molecular mechanical (QM/MM) approach with techniques that search for minimum energy reaction pathways. Multiple pathways were explored together with different possible end states, involving various protein groups as intermediate proton carriers. The results form the basis for the understanding of the sequence of events that define the proton transport mechanism.

P-77 STRUCTURAL CHANGES DURING THE FORMATION OF EARLY INTERMEDIATES IN THE BACTERIORHODOPSIN PHOTOCYCLE

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Bacteriorhodopsin (bR) is a transmembrane protein in the purple membrane of *Halobacterium salinarium*. This protein has a retinal chromophore covalently bound to Lys_{216} through a protonated Schiff base. Upon absorption of light, bR initiates the photocycle that pumps protons across the membrane. The proton translocation is accomplished by a series of proton transfer processes coupled to conformational changes triggered by the photo-isomerization of retinal. Recent X-ray crystallography [1] and FTIR spectroscopy [2] studies revealed that in the early stage of the photocycle the photo-isomerization of retinal induces rearrangement of hydrogen-bond network (HBN) in the binding pocket, which in turn leads to the primary proton transfer. In the present study, the molecular detail of the rearrangement in the early intermediate states of the photocycle is examined by means of *ab initio* quantum mechanical/classical mechanical (QM/MM) calculations and molecular dynamics (MD) simulations [3].

To describe dynamics of the HBN rearrangement, we performed MD simulations of the photo-isomerization of retinal. We found that the photo-isomerization occurs toward the Asp212 direction and two intermediate states appear sequentially in time after the isomerization. Based on structural and spectroscopic properties of those states obtained by the QM/MM calculations, those two states are assigned to the K and KL intermediate states, respectively. In those intermediate states, the polyene chain of retinal is strongly twisted and the N-H bond of the Schiff base points toward Asp212 in K, and toward Thr89 in KL. A significant HBN rearrangement takes place in the K-to-KL transition and the structure of HBN in the KL state is in good agreement with that in the K state observed by the X-ray measurement [1].

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[2] Kandori H. and Scichida Y. *J. Am. Chem. Soc.* 122, 11745 (2000).
[3] Hayashi, S. et al. *Biophys. J.* 83 (2002). In press.

P-78 SPECTRAL TUNING AND PHOTOISOMERIZATION DYNAMICS OF RETINAL IN RHODOPSINS STUDIED BY AB INITIO QUANTUM MECHANICAL/MOLECULAR MECHANICAL CALCULATIONS

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Rhodopsin proteins function as photoreceptors in vision and bioenergetics processes. Although rhodopsin receptors employ the same chromophore, the retinal protonated Schiff base, to detect light, they maximally absorb light at different wavelengths. A longstanding question is how the excitation energy of retinal is tuned by the proteins. Upon the photoabsorption of chromophore, the activation of rhodopsins starts with an extremely fast (200-500 fs) photoinduced isomerization of retinal around a double bond, which is one of the fastest chemical reactions in nature. Although many ultrafast spectroscopic studies have been pursued to monitor the process, molecular detail of the photoisomerization dynamics is still unclear.

In this study, we performed combined *ab initio* quantum mechanical/molecular mechanical (QM/MM) calculations to investigate the spectral tuning and photoisomerization dynamics of retinal in rhodopsins. Recent advance in determination of high resolution x-ray crystallographic structures of two very homologous bacterial rhodopsins, bacteriorhodopsin (bR) and sensory rhodopsin II (sRII), with very distinct spectral maxima at 568 and 497 nm, respectively, enabled us to compute the spectral shift and, thereby, identify the physical mechanism of spectral tuning [1]. The results explained the major part of the observed differences between the spectra of bR and sRII, attributing them to a shift of the G helix which render the distance between the retinal Schiff base and its counterion (Asp201) shorter in sRII. We have also succeeded in simulating *in situ* photoisomerization dynamics of a chromophore analogue in bR by using the *ab initio* QM/MM technique. The present all electron description for the electronic states has revealed a key role of electrostatic interactions of the retinal Schiff base with its counterions in the curve crossing dynamics.

[1] Hayashi S., et al. J. Phys. Chem. B 105, 10124 (2001).

P-79 MOLECULAR DYNAMICS SIMULATION OF EARLY EVENTS IN THE ACTIVATION OF RHODOPSIN

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Retinal *cis-trans* isomerization and early relaxation steps have been studied in a 10 ns molecular dynamics simulation of a fully hydrated model of membrane-embedded rhodopsin. The isomerization, induced by transiently switching the potential energy function governing the C_{11}=C_{12} dihedral angle of retinal, completes within 150 fs and yields a strongly distorted retinal. The most significant conformational changes in the binding pocket are straightening of retinal's polyene chain and separation of its β-ionone ring from Trp265. In the following 500 ps, transition of 6*s-cis* to 6*s-trans* retinal and dramatic changes in the hydrogen bonding network of the binding pocket involving the counterion for the protonated Schiff base, Glu113, occur. Furthermore, the energy initially stored internally in the distorted retinal is transformed into non-bonding interactions of retinal with its environment. During the following ten nanoseconds, increased mobilities of some parts of the protein, such as the kinked regions of the helices, mainly helix VI, and the intracellular loop I2, were observed, as well as transient structural changes involving the conserved salt bridge between Glu134 and Arg135. These features prepare the protein for major structural transformations achieved later in the photocycle. Retinal's motion, in particular, can be compared to an opening turnstile freeing the way for the proposed rotation of helix VI. This was demonstrated by a steered molecular dynamics simulation, in which an applied torque enforced the rotation of helix VI.

P-80 EXCITED STATE MOLECULAR DYNAMICS OF RETINAL MODEL CHROMOPHORES

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The photochemical *cis-trans* and *trans-cis* isomerisation of retinal chromophores necessitate expensive quantum chemical treatment, too costly to treat the complete chromophore. We investigate the dynamics of retinal model chromophores consisting of three double bonds including the positive Schiff base nitrogen. With 6 π-electrons in 6 π-orbitals as ‚active' space of CASSCF based MD-simulations we are able to follow the isomerisation of this model systems. We will present model studies for *cis-trans* and *trans-cis* isomerisations that may provide detailed insight into the dynamics of the initial photochemical event in rhodopsin and bacteriorhodopsin.

P-81 INFLUENCEOF METHYLATION AND BOND TORSION ON THE EXCITED STATE PROPERTIES OF THE PROTONATED SCHIFF BASES $C_5H_8N^+$ AND $C_7H_{10}N^+$. A CASSCF/CASPT2 STUDY

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During the photocycle of the rhodopsin the retinal molecule undergoes many conformational changes. In addition to the *cis-trans* isomerization of the 11-12 bond other bonds are rotated and distorted as a result of the interaction with the protein environment. Understanding how these changes affect the absorption of the chromophore is an important step towards understanding how the protein and its interplay with the chromophore control the rhodopsin photocycle.

Even with modern computer software and resources it is impossible to calculate the excited states of the complete retinal chromophore with sufficient accuracy for spectral correlation. So we take a first step and calculate small model chromophores. It remains to be seen whether the result we obtain can be transferred to the native chromophore.

We have examined the influence of methyl substitution and bond torsion on the protonated Schiff base retinal analogues $C_5H_8N^+$ and $C_7H_{10}N^+$ containing respectively 3 and 4 double bonds. Properties of the ground and the two lowest excited states are calculated based on DFT optimized geometries using CASSCF and CASPT2 methodology which considers dynamic correlation effects.

P-82 *AB INITIO* CALCULATIONS ON RETINAL MODEL CHROMOPHORES INCLUDING EFFECTS OF THE PROTEIN ENVIRONMENT

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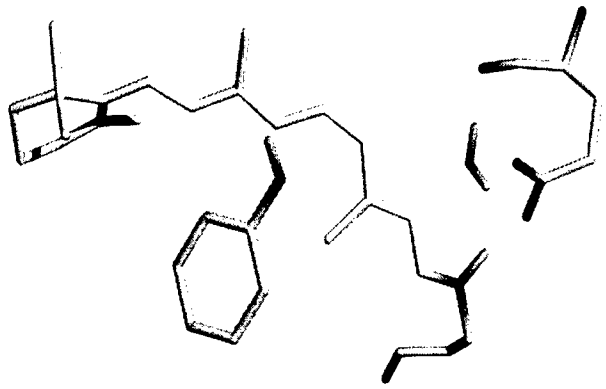
The absorption maximum of a protein bound retinal Schiff base can be anywhere between 425 and 560 nm: a protein-induced spectral shift known as the opsin shift. Understanding the mechanism of wavelength regulation of the chromophore is important for understanding how the protein works which binds the chromophore.

With its unsymmetrical electronic structure and internal distortions retinal is a chal-lenge to any theory attempting to describe the excited states of the molecule. As a step towards this goal we are treating simpler model systems using *ab initio* quantum theory. We have shown recently that theory is able to quantitatively treat the excited state properties (energies and transition moments) of cyanine dyes, hetero substi-tuted symmetrical π-delocalized systems[1,2] as well as symmetric polyenyl cations[3].

Employing a CASSCF reference function corrected by a perturbational treatment (CASPT2) to account for dynamical correlation effects yields quantitative agreement with experiment for systems with 4 to 12 π-electrons.



To simulate interactions between the protein pocket and the chromophore we use this methods to calculate fragments of retinal protonated Schiff bases including parts of the protein environment.

[1]V. Buß, M. Schreiber and M. P. Fülscher, Angew. Chemie Int. Ed. (2001), 3189-3190.
[2]M. Schreiber, V. Buß and M. P. Fülscher, Phys. Chem. - Chem. Phys. (2001), *3*, 3906-3912.
[3]M. Schreiber and V. Buß, Phys. Chem. - Chem. Phys. (2002), *4*, 3305-3310.

P-83 HIGH-QUALITY DFT BASED CALCULATION OF THE CHROMOPHORE BINDNG POCKET OF RHODOPSIN

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Despite the recent progress regarding the structure elucidation of rhodopsin key questions regarding the chromophore and its immediate environment in the protein remain to be answered:

- What is the conformation of the chromophore about the C6-C7 bond: is it *s-cis,* as has been traditionally assumed, or is it *s-trans* as has been proposed recently by Watts and co-workers? Is the C12-C13 bond twisted clockwise or counter-clockwise? This question appears to be settled in favour of the former.
- How is the chromophore kept protonated inside the binding pocket in the presence of strongly basic groups, such as deprotonated E113?

Based on a self-consistent-charge density functional based tight-binding scheme recently developed in Paderborn calculations will be presented which attempt to answer these questions. In summary we conclude that

- Both conformations with the β-ionone ring in the *cis-* and the *trans* conformation fit into the pocket formed by the 27 next-nearest amino acids of the chromophore. Of the two possible 6-*s-cis*-conforamtions the one with a negative dihedral angle is preferred, in agreement with an earlier proposal based on CD-spectral results.
- The chromophore when embedded with a planar conformation from C7 to C15 into the pocket suffers strong non-planar distortion most probably on account of steric interaction with A117. The resulting absolute conformation is in agreement with our earlier assignment.

P-83a THE ROLE OF THR94 IN STABILIZING THE PROTONATED CHROMOPHORE IN RHODOPSIN

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The chromophore of rhodopsin is 11-*cis*-retinal which is bound *via* a covalent Schiff base linkage to the ε-amino group of Lys296. The Schiff base nitrogen is protonated, with Glu113 providing the neutralizing negative charge. The chromophore stays protonated throughout the visual cycle until meta II, the so-called signalling-state, is reached and the proton is transferred from the chromophore to the counterion.

Protonation is of pivotal importance for the chromophore to function properly, though what keeps it protonated in the presence of the strongly basic counterion is not understood at all. Water molecules appear necessary to moderate the hydrogen-bond, but there could be any special interaction of the unsaturated chain with the protein environment which increases the basicity of the conjugated Schiff base.

We have investigated a minimal model for the salt bridge consisting of the chromophore-lysin chain, Glu113, Thr94 and a molecule of water. Coordinates were taken from the recent x-ray structure; water was put in a cavaty near Glu113. Different configurations were studied with molecular dynamics using the Vienna *ab initio* simulation package (VASP). The - preliminary - results are:

- In the complete minimal model the chromophore is protonated (N-H distance 1.10 ± 0.02 Å, total distance N-O: 2.6 Å);
- Without the water and Thr94 the chromophore immediately deprotonates (O-H distance 1.0 Å ± 0.08 Å);
- Without Thr94 (but with water) we observe large-amplitude oscillations of the proton between the two heavy centers;
- Without water (but with Thr94) the proton stays attached to the nitrogen, though the amplitudes are moderately larger.

We conclude that Thr94, by engaging the Glu113 in a hydrogen-bond network, plays a critical role in stabilizing the protonated status of the chromophore in rhodopsin.

P-84 A STRUCTURAL ORIGIN OF THE FLIP OF THE CHROMOPHORE IN THE RHODOPSIN PHOTOCASCADE

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The flip of the β-ionone moiety is an early event of the conformational change of the chromophore in the rhodopsin photocascade. The structure of the bathorhodopsin chromophore generated by molecular dynamics calculation starting from the crystal structure of rhodopsin suggested a twisted conformation of the C_{11}-C_{12} double bond due to a steric interaction between 13-methyl and the main chain carbonyl oxygen of Cys183 The steric interaction of 13-methyl group suggested an ultra fast Hula-twist photoisomerization of the C_{11}-C_{13} portion, followed by a rotation of the C_{11}-N_{ζ} portion in the limited space of the chromophore-binding pocket of rhodopsin. The mode of the twist of the double bond (a negative dihedral angle for C_{10}-C_{11}-C_{12}-C_{13}) in the chromophore model suggested a flip of the β-ionone moiety over TMs 3 and 4 for restoration of the relaxed double bond in the following transition. The outward swing of the C-terminus of TM3 and the simultaneous swing of the N-terminus of TM4 allowed the chromophore to form a putative lumirhodopsin structure with all relaxed double bonds. The structure model of the lumirhodopsin chromophore showed a close contact between the 9-methyl group and Trp296, suggesting a further motion of TMs 3 and 4 for a more relaxed structure, which would lead to a structural model of metarhodopsin I.

P-85 QUANTUM CHEMICAL STUDY ON THE ABSORPTION MAXIMA OF RHODOPSIN AND HALORHODOPSIN

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A major issue in the retinal protein photochemistry is to elucidate the origin of the spectral shifts, induced by transfer of the chromophore from the solution state to the protein-binding state. In this study, the absorption maxima of rhodopsin (Rh) and halorhodopsin (HR) were calculated using the QM/MM-CI (quantum mechanics/molecular mechanics-configuration interaction) method recently developed by our group to interpret the spectral shift in bacteriorhodopsin. The initial atomic coordinates of these proteins were taken from the corresponding X-ray structures, followed by geometry optimization calculations. For Rh, it was found that the absorption maximum depends on the ionization state of Glu181. When it is assumed to be protonated, the calculated absorption maximum was 502 nm, in excellent agreement with the experimental value (498 nm). In addition, the calculation successfully reproduced the absorption maxima of several mutants. For HR, it has been reported that the absorption maximum exhibits a 10 nm red shift, accompanied by binding of chloride ion to the protein, despite that the ion is located in the vicinity of the Schiff base nitrogen of the chromophore, namely in the counterion position. Obviously, the direction of the observed shift is opposite to that induced by the usual counterion effect. We indicate that such an abnormal spectral shift can be explained by taking into account the electronic polarization of the protein environment upon excitation of the chromophore.

P-86 A MOLECULAR DYNAMICS SIMULATION OF PHOTOEXCITATION OF BOVINE RHODOPSIN AND THE GECKO PIGMENTS P467 AND P521: THE IDENTIFICATION OF VIBRATIONAL MODES PARTICIPATING IN THE INITIAL PHOTOACTIVATION PROCESS

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The initial photoexcitation process in three vertebrate visual pigments, bovine rhodopsin and gecko P467 and P521, was analyzed with molecular dynamics simulations. Each pigment considered represents a distinct 11-*cis* retinal-binding photoreceptor class: P467 and P521 are members of the RH2 ("Rhodopsin-like opsins") and M/LWS ("Medium/Long Wave Sensitive opsins") visual pigment families, and provide contrast to the archetypical bovine rhodopsin. The P467 and P521 structures were derived by threading the respective peptides onto the backbone of the x-ray diffraction structure of bovine rhodopsin. Molecular dynamics trajectories for the three structures were run with the Enzymix force field. A change from the ground to the excited state was simulated by a redistribution of electrostatic charge within the chromophore. The time-dependent energy gap between the states, calculated during separate trajectories in the ground and excited states, reflects structural fluctuations of the protein and chromophore. From the mean values of the energy gaps, we can calculate the contributions of the proteins to the excitation energy and the reorganization energy. The Fourier transform of the energy gap autocorrelation function then reveals the molecular oscillations that are coupled to the simulated state change. These modes can be associated with known characteristic vibrational frequencies, and may allow us to identify some of the key residues and groups that influence the excitation in each of the three types of visual pigments.

P-87 ASCIDIAN ARRESTIN (Ci-Arr), THE ORIGIN OF THE VISUAL AND NONVISUAL ARRESTINS OF VERTEBRATES

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Arrestin is one of the key proteins for the termination of G protein signaling. Vertebrates possess two types of arrestins; visual arrestin expressed exclusively in photoreceptor cells in retinae and pineal organs and β-arrestin expressed ubiquitously. Unlike visual arrestin, β-arrestin contains the clathrin-binding domain (CBD) at the C-terminal that is responsible for the agonist-induced internalization of GPCRs.

We isolated a novel arrestin gene (*Ci-arr*) from larvae of the ascidian *Ciona intestinalis*, the primitive chordate. Interestingly, Ci-Arr has characteristics of both visual and β-arrestin. Like visual arrestins, the expression of *Ci-arr* was restricted to the photoreceptors in the larva. However, Ci-Arr contained CBD and promoted the GPCR internalization in HEK293tsA201 cells like β-arrestins. The phylogenetic tree shows that Ci-Arr is branched from a common root of visual and β-arrestins. Southern analysis suggests that the *Ciona* genome does not contain any other arrestin gene. These results suggest that the visual and β-arrestin genes were generated by duplication of the prototypical arrestin gene like *Ci-arr* in the early evolution of vertebrates.

Immunohistochemical analyses using the anti-Ci-Arr antibody revealed the detailed structure of the photoreceptors in the tadpole-like larva, including the outer segments, the cell bodies, axons and nerve terminals. Lack of layered structure suggests that the ocellus of the larva is similar to that of pineal organs rather than lateral eyes of vertebrates. We also investigated the morphological changes of the photoreceptors during larval development. One hour after hatching, the cell bodies were fully developed, whereas the axons were still immature. Then their axons gradually extended. After 4 hours, the extensionof axons stopped and the nerve terminals started growing. The morphological changes of the photoreceptors seem to be consistent with development of the photic response of the larvae.

P-88 MOLECULAR MECHANISMS OF SPECTRAL TUNING OF THE NEWT SWS2 PIGMENT

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Based on their amino acid sequences, the vertebrate visual pigments can be classified into 5 groups, RH1, RH2, SWS1 (Short Wavelength Sensitive1), SWS2, and M/LWS (Mid/Long Wavelength Sensitive). Each pigment which belongs to a given group has a similar absorption maxima within a broad range. There are highly conserved amino acids in the sequences, especially around the ring of the retinal chromophore, the Schiff base and the counter ion.

We have isolated the cDNA which codes newt SWS2 pigment and reported the amino acid sequence, and distribution of this pigment in the different types of photoreceptor cells in the newt retina [Takahashi, Y. et al., FEBS Letters, 2001, 501, 151-155]. The absorption maximum of the reconstituted newt SWS2 pigment (474nm) is greatly red shifted compared to other known SWS2 pigments (430-455nm). In order to determine the key amino acid residues that control spectral tuning for the newt SWS2 pigment, we replaced several uniqueamino acid s that differ between newt and bullfrog SWS2 pigment (lambda max = 430nm); P91S, S94A, I122M, C127S, S211C, Y261F, and A292S. Some of these site directed mutants led to blue shifts of the newt pigment and a multiple all mutant was blue shifted to 432nm. There is a 44nm difference between the newt and frog SWS2 pigment; it is likely that the 7 amino acids which we replaced are the origin of the red shift of newt SWS2 pigment compared to the frog SWS2 pigment.

P-89 SPECTRAL TUNING OF ULTRAVIOLET AND BLUE ABSORBING VISUAL PIGMENTS IN INVERTEBRATES

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To identify residues important for regulating the absorption differences between the ultraviolet (UV) and short-wavelength (SW) subfamilies of invertebrate rhodopsins, we performed a sequence alignment of the known members of these families of invertebrate visual pigments. We identified 27 residues that were conserved within the UV and SW groups, but that differed between them. We further restricted these sites to include only amino acids that differed in charge or polarity. Based on this analysis, we constructed a series of nine mutant genes encoding variants of the *Drosophila* Rh5 pigment (a SW pigment with λ_{max} = 437 nm) containing single or multiple amino acid changes corresponding to those within the *Drosophila* Rh3 pigment (a UV pigment with λ_{max} = 345 nm). The modified genes were expressed in the R1-6 photoreceptor cells of *y w; ninaE* mutant flies. We characterized the spectral sensitivity of the transgenic flies electrophysiologically. We found that of the nine constructs and twelve amino acids that were modified, all but one caused little or no change in the spectral sensitivity profile of the animals expressing them. Interestingly, we found that a single amino acid change Rh5 N104K, caused a dramatic 52 nm blue shift, in the spectral sensitivity of animals expressing this construct. These findings suggest that the introduction of a lysine residue at this position in the invertebrate UV pigments is responsible for a large part of the spectral difference between the SW and UV subfamilies of invertebrate pigments. This position corresponds to G90 in bovine rhodopsin, a residue in close proximity to the chromophore. Amino acids at this position have been shown to play a role in the tuning of avian UV and violet visual pigments. The introduction of a positively charged lysine at this position would be expected to disrupt the interaction between the neighboring counter-ion and the positively charged Schiff's base, and potentially produce a pigment having a dramatically blue shifted absorption maximum.

P-90 THE MOLECULAR MECHANISM FOR THE SPECTRAL SHIFTS BETWEEN VERTEBRATE ULTRAVIOLET- AND VIOLET-SENSITIVE CONE VISUAL PIGMENTS

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The short wave-sensitive visual pigments of vertebrate cone photoreceptors are divided into two classes on the basis of molecular identity, SWS1 and SWS2. Only the SWS1 class are present in mammals. The SWS1 pigments can be further sub-divided into violet sensitive (VS) with λ_{max} values generally between 400 and 430 nm, and ultraviolet sensitive (UVS) with λ_{max} values <380 nm. Phylogenetic evidence indicates that the ancestral pigment was UVS and that VS pigments have evolved separately from UVS pigments in the different vertebrate lineages.

Amino acid sequence comparisons of the UVS pigments of teleost fish, amphibia, reptiles and mammals shows that site 86 (bovine rod opsin numbering) is invariably occupied by Phe but it is replaced in bovine and porcine VS pigments by Tyr. Using site-directed mutagenesis of goldfish UVS opsin, we have shown that a Phe86Tyr substitution is sufficient by itself to shift the λ_{max} of the goldfish pigment from a wild type value of 360 nm to around 430 nm, and the reverse substitution of Tyr86Phe into bovine VS opsin produces a similar shift in the opposite direction. The substitution of this single amino acid is sufficient to account therefore for the evolution of bovine and porcine VS pigments. The replacement of Phe with polar Tyr at site 86 is consistent with the stabilisation of Schiff base protonation in VS pigments.

Site 86 is also substituted in bird, amphibian and primate VS pigments where it is occupied respectively by Ser, Met and Leu. However, substitution of Phe86 in goldfish with any of the latter residues is without effect on the λ_{max} of the regenerated pigment, indicating that Ser, Met and Leu substitutions at site 86 are alone insufficient to account for the longwave shift of the VS pigments of birds, amphibia and primates.

P-91 COUNTERION SWITCHING DURING PHOTOACTIVATION OF RHODOPSIN

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We have obtained experimental evidence that the Schiff base counterion switches from Glu113 to Glu181 during the photoactivation of rhodopsin. Biochemical studies showed that Glu181 mutation alters the decay of Meta II, the reactivity of rhodopsin toward hydroxylamine, and the rate of transducin activation (Yan et al., *Biochemistry*, 41, 3620, 2002). Raman spectra of Glu181 mutants in the rhodopsin state and in the Batho state identified perturbations of the protonated Schiff base (PSB), whose magnitude is consistent with a neutral (protonated) Glu181 residue. Time-resolved spectroscopic studies demonstrated that Glu181 mutagenesis perturbs the early photo-kinetics such as the decay of Batho, BSI and Lumi. These experimental results together with a recent time-resolved Raman study, which showed abrupt changes in the H-bond environment during the BSI-Lumi-Meta I transition (Pan et al., *Biochemistry*, 40, 7929, 2001) encouraged us to examine the role of Glu181 in rhodopsin. We thus studied the pH dependence of E181Q of the Meta I state revealing an acid/base equilibrium of the SB near physiological pH. This pH dependence of the Meta I state was not observed in parallel experiments for E113Q and the wild type pigment, but exhibits very similar behavior to that of E113Q in the dark state. We conclude that Glu181 is the counterion of the PSB in the Meta I state, and that the counterion switches from Glu113 to Glu181. Based on these observations and the published crystal structures, we speculate that a proton is transferred from Glu181 to Glu113 through a H-bond network extending across the binding pocket. In particular, residues situated on β4 such as Ser186, Cys187, and Tyr192 are important in the H-bond network. It is known that β4 is highly conserved in vertebrate visual pigments but is variable in other classes of GPCR. Our studies suggest that the specific role of β4 in rhodopsin is to provide residues on a flexible loop-structure to form a H-bond network, which evolves during the photoactivation and facilitates the counterion switch.

P-92 ACTIVE STATE PHOTOPRODUCT IN 11-*CIS*-LOCKED RHODOPSIN

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The visual pigment rhodopsin is characterized by an 11-*cis* retinal chromophore bound to Lys296 via a protonated Schiff base. Following light absorption the C_{11}=C_{12} double bond isomerizes to *trans* configuration and triggers protein conformational alterations. These alterations lead to the formation of an active intermediate (Meta II) which binds and activates the visual G protein, transducin. We have examined by UV-vis and FTIR spectroscopies the photochemistry of a rhodopsin analogue with an 11-*cis*-locked chromophore, where *cis* to *trans* isomerization around the C_{11}=C_{12} double bond is prevented by a 6-membered ring structure ($Rh_{6.10}$). Despite of this lock, the pigment was found to be capable of forming an active photoproduct with a characteristic protein conformation similar to that of native Meta II. This intermediate is further characterized by a protonated Schiff base and protonated Glu113, and by its ability to bind a transducin derived peptide which was previously shown to interact efficiently with native Meta II. The yield of this active photointermediate is pH-dependent and decreases from about 50% at pH 5.0 to less than 10% at neutral pH. This study shows that with the C_{11}=C_{12} double bond being locked, isomerizations around the C_9=C_{10} or the C_{13}=C_{14} double bonds may as well lead to an activation of the receptor.

P-93 DISRUPTION OF IONIC LOCKS DURING RHODOPSIN ACTIVATION EQUIVALENCE OF PROTONATION AND ION BINDING

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We studied the influence of salts on the pH-dependent conformational equilibria between the active and the inactive photoproduct states of rhodopsin, Meta II and Meta I, respectively, and between the active and inactive conformations of the apoprotein opsin. In both equilibria the active, low-pH species is favored in the presence of medium to high concentration of salt. The ion selectivity for the Meta I/Meta II equilibrium is particularly pronounced for the anions and follows the series trichloroacetate > thiocyanate > iodide > bromide > sulfate > chloride > acetate. The Hill coefficient of this salt induced transition is close to 2.0. Both ion selectivity and Hill coefficient suggest that the transition is mainly regulated by ion binding to two specific charged binding sites in the protein with smaller contributions being due to the Hofmeister effect. We propose that these putative ion binding sites are identical to those sites that are titrated in the corresponding pH-dependent conformational transition. They presumably function as ionic locks, which keep the receptor in an inactive conformation, and which may be disrupted either by pH-dependent protonation or by salt-dependent ion binding. Such interhelical constraints are expected to be localized between the E(D)RY motif on the cytoplasmic terminus of helix 3 and the cytoplasmic end of helix 6 for the Meta I/Meta II equilibrium, and between Glu-113 on helix 3 and Lys-296 on helix 7 for the opsin equilibrium.

P-94 CHARACTERIZATION OF RHODOPSIN EXCITATION INTERMEDIATES WITH TIME-RESOLVED OPTICAL ABSORBANCE MEASUREMENTS

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Rhodopsin crystals have dispelled much of the darkness surrounding the static structure of visual pigments. However, because the energetics which stabilize the static rhodopsin structure are poorly understood, extrapolation of purely structural results into a mechanism of receptor activation cannot proceed very far. The real significance of the crystal structure for receptor activation comes from using it as a guide to the interpretation of data from traditional biophysical techniques, and strong synergism exists with biophysical techniques having the most dynamical content. In particular, transient absorbance measurements which have excellent time resolution but which are open to criticism for low "information content" have benefited from the wealth of detail supplied by the crystal structure and are well positioned to help animate it.

The absolute conformation of single bond twists deduced from the crystal structure combined with previous results from artificial rhodopsin pigments give a much more concrete picture of bathorhodopsin (Batho). Decay of Batho to the next blue shifted intermediate (BSI) primarily involves intrachromophore barriers and little protein change. However, BSI formation sets the stage for the first protein change, a change which can only be understood when a more concrete picture of the BSI chromophore is developed. Artificial rhodopsins made from synthetic retinal analogs have been used to better characterize BSI and its subsequent decay to lumirhodopsin (Lumi).

Transient absorbance measurements on rhodopsin mutants have been used to better understand the interaction of the chromophore with its counterion and residues in EC Loop II which are adjacent to it. Previous work on gecko P521 which showed that chloride affects Batho decay, can now be better understood since the LW cone pigment chloride binding site is known to be near the chromophore in EC Loop II.

P-95 TIME-RESOLVED RESONANCE RAMAN ANALYSIS OF CHROMOPHORE STRUCTURAL CHANGES IN THE BSI, LUMI AND META I INTERMEDIATES

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A microchip flow technique has been used to obtain time-resolved resonance Raman spectra of rhodopsin's BSI, Lumi and Meta I intermediates at room temperature to elucidate the structural changes of the chromophore and its interaction with the protein.[1,2] Transient Raman spectra of BSI, Lumi and Meta I at 250 ns, 16 μs and 1 ms delay times, respectively, are obtained using a microprobe system to focus displaced pump and probe laser beams in a microfabricated flow channel. BSI exhibits relatively intense HOOP modes at 886 and 945 cm^{-1} that are assigned to $C_{14}H$ and $C_{11}H{=}C_{12}H$ A_u wags, respectively. This result suggests that in the transition of Batho to BSI the highly strained all-trans chromophore has relaxed in the C_{10}-$C_{11}{=}C_{12}$-C_{13} region, but is still distorted near C_{14}. The fingerprint modes of both Lumi and Meta I are very similar and characteristic of an all-trans chromophore, indicating that the transitions from BSI-to-Lumi and Lumi-to-Meta I involve a relaxation of the chromophore to a more planar all-trans conformation and the elimination of the structural perturbation that uncouples the 11H and 12H wags in Batho. The $C{=}NH^+$ stretching mode in BSI at 1653 cm^{-1} exhibits a normal deuteration induced downshift of 23 cm^{-1}, implying that there is no significant structural rearrangement or alteration of the Schiff base counterion region in the transition of Batho to BSI. Surprisingly, the 1638 cm^{-1} $C{=}NH^+$ stretching mode in Lumi is unusually low and shifts only 7 cm^{-1} in D_2O, suggesting a dramatic weakening of the interaction of the Schiff base group with its counterion in the BSI-to-Lumi transition. The structural relaxation of the chromophore-protein complex in the BSI-to-Lumi transition thus appears to drive the Schiff base group out of its hydrogen-bonded environment near Glu113. However, the $C{=}NH^+$ stretching mode in Meta I is found at 1654 cm^{-1} and exhibits a normal deuteration induced downshift of 24 cm^{-1}, identical to that of the all-trans protonated Schiff base. *Based on these data we proposed that there is a switch of the Schiff base counterion between BSI and Meta I and that Lumi is the transition state between these two forms.*[1,2]

1. Pan, D.; Mathies, R. A. *Biochemistry* **2001**, *40*, 7929-7936.
2. Pan, D.; Ganim, Z.; Kim, J. E.; Mathies, R. A. *J. Am. Chem. Soc.* 2002, 124, 4857-4864.

P-96 DECAY OF PHOTOACTIVATED RHODOPSIN IN ROD DISC MEMBRANES: META III STORAGE FORM AND ACTIVE OPSIN-ALL-*TRANS*-RETINAL COMPLEX

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After light-induced *cis/trans* isomerization of its chromophore, rhodopsin forms the active Meta II intermediate, in which the Schiff base linkage of the all-*trans*-retinal to Lys^{296} is intact but deprotonated. The subsequent hydrolysis of the retinylidene bond and release of the photolyzed all-*trans*-retinal (*atr*) from the chromophore pocket is mandatory for regeneration of the pigment with metabolically supplied 11-*cis*-retinal. The classical scheme for the decay of Meta II comprises two parallel pathways, one leading into opsin and free all-*trans*-retinal, the other into an intermediate, so-called Meta III, which remains in equilibrium with Meta II. Using a fluorescence assay for the retinal release and UV/Vis spectroscopy, we have reinvestigated these conversions on isolated disc membranes.

Release of *atr* from its binding pocket was monitored by the opsin fluorescence increase during the decay of Meta II. The final level of the fluorescence change is twice as large at pH 6, as compared to pH 8, indicating that at least two species contribute to the final level. A comparison with UV/Vis spectra recorded during the decay reaction shows that this phenomenon reflects the pH-dependent formation of an intermediate with λ_{max} = 460-470 nm. The data show that in this species, *atr* is retained in the retinal pocket, thus persistently quenching the opsin fluorescence. By its absorption and persistent fluorescence quench, we term this species Meta III.

Meta III production was prevented by addition of $G_t\alpha$-high affinity analogue-peptide, which mimics G_t binding with specificity for the Meta II conformation. Under these conditions, both time course and amplitude of the fluorescence increase are essentially pH independent between pH 6 and 8, suggesting independence of retinylidene hydrolysis on bulk pH.

The opsin/*atr* complex generated by decay of Meta II is active against G_t. It is likely to represent the previously described non-covalent complex between opsin and exogenously added *atr*. During the decay reaction, neither the *atr* in this complex nor added excess of *atr* inhibit regeneration of rhodopsin with 11-*cis*-retinal. Additionally, the presence of NADPH, which is a necessary cofactor for the production of all-*trans* retinol from *atr* by retinol dehydrogenase, did not accelerate the Meta II decay process and, notably, did not change the amount of Meta III formation.

Our data obtained with isolated disc membranes are consistently described by a reaction scheme comprising two parallel and quasi-irreversible pathways, namely:

i) the persistent formation of a storage form, which is inaccessible to regeneration by 11-*cis*-retinal (Meta III) and

ii) the eventual release of the photolyzed chromophore through an active conformation (the *atr* opsin complex).

P-97 RHODOPSIN ELECTROSTATICS IN THE NIGHT BLINDNESS MUTANT G90D

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While most rod opsin mutations in humans cause photoreceptor degeneration, a few (G90D, T94I and A292E) promote a state of rod desensitization called congenital stationary night blindness (Dryja et al., 1993; Sieving et al., 1995; al-Jandel et al., 1999). The sidechains of the mutated residues inhabit the immediate environment of the protonated Schiff base (PSB) in the crystal of ground state rhodopsin (Palczewski et al., 2000). With early receptor current (ERC) assays we investigated the electrical events associated with late conformational activation of expressed human G90D rhodopsin. In wild type (WT) rhodopsin successive flashes progressively deplete and eventually extinguish the outward msec-order R_2 charge motion associated with Metarhodopsin-II formation. Unlike WT and mutant pigments investigated to date, G90D is unique in that a photostationary state (PSS) is established upon successive 500 nm flashes. On the first flash G90D generates a brief outward R_2 charge motion. The second and third flashes lead to progressive and complete inversion of R_2 charge motion to populate a PSS that resists charge extinction (bleaching) upon further stimuli. The inward R_2 of the G90D PSS is similar to the R_2 signal of E113Q or WT at low extracellular pH (pH_o). While WT R_2 is sensitive to pH_o with two titrations (outward pK_a = 7.0, inward pK_a ~4.5), the pH_o sensitivity of G90D PSS has only an anomalous inward titration. The PSS senses intracellular pH (pK_a 6.8) but the dynamic range is compressed relative to WT and there appears to be little effect of pH_i on R_2 relaxation rate. Voltage sensitivity of WT pigment is lost in the G90D PSS. Regeneration of G90D with all-*trans*-retinal simulates the inward charge motion of the PSS established from an 11-*cis*-retinal regenerated pigment, but the former bleaches readily. The PSS action spectra of G90D regenerated with 11-*cis*-retinal (peak 437 nm) is similar to the spectra of G90D regenerated with all-*trans*-retinal (peak 450 nm). This suggests that the PSS has an all-*trans*-retinylidene chromophore with a PSB linkage. We hypothesize that the G90D PSS in membranes relates to chromophore isomerization, a stable shift in PSB counterion from E113 to G90D, and a resultant PSB environment that has protection from hydrolysis and/or a high quantal efficiency of photoconversion. The PSS may relate to elevated rod sensitivities in patients with G90D.

P-98 THERMODYNAMICS OF PHOTOREACTION IN BOVINE RHODOPSIN: A PRELIMINARY PHOTOACOUSTIC MEASUREMENT

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Using pulsed time-resolved photoacoustic spectroscopy we have previously measured the thermodynamic parameters, e.g., the molecular volume change (conformational change), enthalpy, and entropy changes associated with the individual photoreaction steps, of proton transfer in bacteriorhodopsin (BR) and electron transfer reaction in photosynthesis. In this study, our preliminary photoacoustic data on bovine rhodopsin (Rho) excited at 500 nm revealed a large apparent volume change ($+33\pm2A^3$) in the early step of photoreaction on the ~1 microsecond time scale. The observed reaction was tentatively assigned to the formation of Lumi from Rho*. Assuming the quantum yield is 0.67, the conformational change of this early step would be $+49\pm2A^3$, which is consistent with that ($\sim+50A^3$) reported previously [Strassburger JM, Gartner W, and Braslavsky SE (1997) *Biophysical J.* 72: 2294-2303]. However, this value is dramatically different from that ($\sim+3A^3$) of the early step of the photocycle in BR [Zhang D and Mauzerall D (1996) *Biophysical J.* 71: 381-388], a proton pump, even though both have similar structures of seven transmembrane helices and the same chromophore molecule, retinal. If the quantum yield is the same at ~4C and 25C, the apparent enthalpy change of the Rho* $\rightarrow$ Lumi step in Rho was found to be unexpectedly high, even higher than the energy of the exciting light. These data support the view that Rho* in the visual system functions as a "trigger". Upon light excitation, Rho may undergo a fast reaction associated with large positive conformational change and large heat release on the <microsecond time scale. In contrast BR* stores some of its energy for proton pumping and the large conformational volume change occurs later. Further studies will be focused on the other steps of photoreaction in native and mutant Rho from nanosecond to millisecond time scales.

P-99 THE PHOTOBLEACHING SEQUENCE OF VERTEBRATE ULTRAVIOLET VISUAL PIGMENT

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Cone pigments in the SWS1 group mediate visual sensitivity in the short wavelength region of the spectrum, exhibiting absorption maxima between 350-450 nm, the widest energy range in the opsin classes. Moreover, SWS1 pigments are the only opsins with absorption maxima blue-shifted compared to a protonated retinal Schiff base in aqueous solution, (~440 nm). Short-wavelength mouse UV (MUV) cone opsin was expressed in mammalian COS1 cells and regenerated with 11-cis retinal in delipidated form. The photobleaching pathway of MUV was studied by using cryogenic UV-Visible and FTIR difference spectroscopy. MUV has an absorption maximum at ~357 nm at room temperature and red shifts to ~359 nm with a shoulder at 380 nm at 75K. Upon illumination at 75K, the batho intermediate (~380 nm) forms and thermally decays to lumi (~440 nm). A blue-shifted intermediate, BL (~375 nm) is observed prior to lumi at temperatures greater than 180K which is not observed in the other cone pigment photobleaching pathways. Decay of the lumi intermediate forms meta I (~480 nm) which decays to form to meta II (~380 nm) at 248K. This pigment follows a different molecular mechanism compared to rhodopsin and (*Xenopus laevis*) violet cone opsin. The mechanism is based on an unprotonated chromophore in the dark. This conclusion is supported by differential FTIR analysis of the 1100-1500 cm^{-1} region. Significant protein conformational changes occur in the batho and lumi intermediates of MUV. The MUV chromophore is initially unprotonated and eventually becomes protonated during the photobleaching cascade.

P-100 ELUCIDATION OF THE NATURE OF THE CONFORMATIONAL CHANGES OF THE EF-INTERHELICAL LOOP IN BACTERIORHODOPSIN AND OF THE HELIX VIII ON THE CYTOPLASMIC SURFACE OF BOVINE RHODOPSIN

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The conformation of the AB- and EF-loop on the cytoplasmic surface of bacteriorhodopsin and of the 4th loop (helix VIII) of bovine rhodopsin were assessed by a combination of time-resolved fluorescence depolarization and site-directed fluorescence labeling. The fluorescence anisotropy decays were measured employing a tunable Ti:sapphire laser/microchannelplate based single-photon counting apparatus with picosecond time resolution. This method allows to measure the diffusional dynamics of the loops directly on the nanosecond time scale. We implemented the method to study model peptides and 2-helix systems representing sequences of bacteriorhodopsin. Thus, we systematically analyzed the anisotropic behavior of the fluorescent dyes (Iodoacetamido-fluorescein, Lucifer Yellow-iodoacetamide, Tetramethylrhodamine-iodoacetamide or Texas Red-bromoacetamide, respectively) covalently bound to a single cysteine on the protein surface and assigned the anisotropy decay components to the modes of motion of the protein and its segments. A conformational change of the EF-loop is expected from the outward tilt of helix F during the bacteriorhodopsin photocycle. The exact nature of this conformational change is not clear. We provide evidence for a surface potential change based switch between two conformational states of the EF-loop, detected with the fluorescent dye bound to position 160. Our results support the theory that the switch of the EF-loop is the missing link between the movement of helix F and the transient surface potential change detected during the photocycle of bacteriorhodopsin.

P-101 PHOTOCHEMICAL AND STRUCTURAL PROPERTIES OF THE RING FUSED ISORHODOPSIN ANALOGS

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Two 9-*cis* retinal analogs, the C_{11}-C_{13} and C_{12}-C_{14} 5-membered ring-fused retinal derivatives, were designed to investigate the property of the chromophore-binding site of rhodopsin. Both of them formed the pigments with apoprotein opsin (λ_{max} = 480 and 509 nm, respectively). The C_{11}-C_{13} ring-fused analog was photoisomerized to all-trans form (λ_{max} >400nm) at –196 °C and the Schiff base was kept protonated even at room temperature. The C_{12}-C_{14} ring-fused analog was photochemically converted to a bathorhodopsin-like product at –196°C and decayed to the deprotonated Schiff base at 0°C. The model building study suggested that the transmembrane helices have to move outward in order to accommodate the retinal analogs. The molecular dynamics simulation of the isomerization of the analog chromophores provided a twisted C_{11}-C_{12} double bond for the C_{12}-C_{14} ring-fused analog and all relaxed double bonds with a highly twisted C_{10}-C_{11} bond for the C_{11}-C_{13} ring-fused analog. The structural model of the C_{11}-C_{13} ring-fused analog chromophore showed a characteristic flip of the cyclohexenyl moiety toward TM3 and 4. The structural models well explain the spectroscopic features of the ring-fused isorhodopsin analogs. These results suggest the possibility that the extra portion of the retinal analog expands the binding pocket and, as a result, the protein moiety takes a conformation similar to that of lumi or a later intermediate.

P-102 RHODOPSIN STRUCTURAL CHANGES IN EARLY AND LATE STATES OF PHOTOLYSIS DETECTED BY VIBRATIONAL CHANGES OF CYSTEINE

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For furthering our understanding of the G protein activation mechanism by rhodopsin, we have tried to detect structural changes of the transmembrane region of rhodopsin by the Fourier transform infrared spectroscopy with the aids of site-directed mutagenesis. For this purpose, we prepared site-directed mutants of rhodopsin where each of the amino acid residues at positions from 122 to 134 in the helix III was replaced with cysteine to use a cysteine S-H stretching change as a probe for the micro-environmental change By using the low temperature FTIR spectroscopy, we detected three cysteine changes upon Batho formation. These residues at positions 117, 118 and 122 were all located near the retinal chromophore. In the Meta II state, additional S-H groups at the positions 123 and 185 changed their vibrational features, whereas the vibrational changes at positions 117 and 118 were restored. In contrast, the introduced cysteines at the cytoplasmic side of the helix III did not change their S-H stretching vibration in both the intermediates. Structural changes occurring in helix III are discussed on the basis of the present FTIR observations.

P-103 STRUCTURAL CHANGES IN LUMIRHODOPSIN AND METARHODOPSIN I STUDIED BY THEIR PHOTOREACTIONS AT 78 K

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Rhodopsin has been considered to change its global protein structure for activation of a G protein transducin. A photoaffinity labeling experiment reported that formation of Lumi accompanied flip-over of β-ionone ring of the retinal chromophore, so that the ring portion was attached to Ala169 of helix IV [Borhan et al. (2000) *Science 288*, 2209-2212]. According to the crystal structure of bovine rhodopsin, the distance between the labeled C3 atom of the chromophore and Ala169 is >15 Å [Palczewski et al. (2000) *Science 289*, 739-745]. These facts suggest that global protein structural changes including helical motions would take place in Lumi. In the present study, Lumi and Meta I are illuminated at 78 K, and protein structural changes are probed by Fourier-transform infrared (FTIR) spectroscopy. We found that Lumi can be photoconverted to rhodopsin at 78 K from the IR spectral analysis of the photoproducts of Lumi. In contrast, more complex spectra were obtained for the photoproducts of Meta I at 78 K, implying that the protein structure of Meta I is considerably altered so as not to be reverted to the original state at 78 K. Thus, the present photoreaction experiments of Lumi and Meta I at 78 K revealed the occurrence of global protein structural changes in the process between them.

P-104 PHYLOGENETIC AND FUNCTIONAL CHARACTERIZATION OF OPSIN FAMILY: CDNA CLONING OF AMPHIOXUS OPSINS AND THEIR EXPRESSION IN CULTURED CELLS

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More than 200 opsins, visual pigments and their related retinal proteins, have been cloned and they have been classified into at least five subgroups based on their amino acid sequence similarity. To investigate the functional diversity of opsin family, we isolated six opsin genes (AmphiRh1-6) from amphioxus and carried out the phylogenetic and biochemical analysis. The molecular phylogenetic tree suggested that each of them is closely related to invertebrate opsins which couple with Gq-type G-protein (Gq-coupled opsin), scallop Go-coupled opsin, mammalian peropsin and encephalopsin which is related to vertebrate Gt-coupled opsin, suggesting that amphioxus possessed comparable number of opsin subtypes to human. We successfully expressed the protein moieties of amphioxus homologs of Go-coupled opsin and peropsin and investigated their characteristics. To our knowledge, this is the first report of the expression of invertebrate "rhodopsin", Go-coupled opsin and peropsin homologues in cultured cells. Together with these results, the relationship between the phylogenetic classification and the molecular properties of opsin will be discussed.

P-105 FULLY FUNCTIONAL VISUAL PIGMENTS IN A 'BLIND' MAMMAL

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The blind mole rat, belonging to the *Spalax ehrenbergi* superspecies or Spalacids, is a unique rodent that spends most of its lifetime underground. The visual system of Spalacids exhibits natural degeneration accompanying thirty million years of adaptation to a subterranean lifestyle. The eyes have been reduced to a very minute size (< 1mm) and are located in the hypertrophied Harderian gland, covered by skin and fur. The anterior eye segments, lens and cornea, start to degenerate early in development. The neuronal components of the visual system are regressed or completely absent. In contrast, bilateral projections from the retina to the suprachiasmatic nucleus (SCN) have been expanded in comparison to sighted rodents and *Spalax* exhibits photoregulation of circadian physiology. All available evidence indicates that *Spalax* is visually blind. Nevertheless, two visual pigments, a rod-like and a green cone-like and, have recently been cloned from *Spalax* eye. Here we present a photochemical and functional characterization of the corresponding recombinant pigments, obtained through baculovirus expression and IMAC purification. Both recombinant pigments show the characteristic propeties of their subfamily (absorbance band, decay of Meta II) and appear to be fully functional up to activation of the downstream effector transducin. The possible relevance of these findings for the evolutionary adaptation of *Spalax* to a subterranean ecotope is discussed.

P-106 MASS SPECTROMETRIC ANALYSIS OF IMMUNOAFFINITY PURIFIED NATIVE AND RECOMBINANT PHOTOPIGMENTS

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G-protein coupled receptors are a subject of much current interest in biomedical investigation. However, G-protein coupled receptors produced by expression systems, due to the low efficiency of their expression and the limited quantity of the expressed to the total amount of the membrane proteins, have evaded mass spectrometric analysis.

The methodology previously developed in our laboratory for the mass spectrometric analysis of these receptors required proteins that were obtained relatively pure and in relatively large quantity from their native membranes. This methodology made it possible to analyze and map the sequence and characterize and quantitate the post-translational modifications (phosphorylation, glycosylation, acetylation and palmitylation) of bovine, rat, porcine, mouse and salamander dim-light sensitive photopigments.

Recently, in order to promote the characterization of cone photopigments, a modified methodology has been developed and applied to the analysis of subnanomolar samples of bovine rhodopsin purified by affinity chromatography, both from native membranes and from a COS cell expression system. In both cases, complete sequence coverage was obtained; the posttranslational modifications were detected and identified. The glycosylation pattern of the native and expressed rhodopsin was found to be different, but.all other modifications, including phosphorylation, were similar.

This methodology will enable mass spectrometric analysis of subnanomolar levels of both rod and cone photopigments, other G-protein coupled receptors and integral membrane proteins, either from their native membranes or as products of expression systems.

Funding provided by NIH EY-04939 and EY-08239, the ONR, and FPB.

P-107

CONSTITUTIVELY AND DARK ACTIVE MUTANTS OF A UV CONE PIGMENT

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We have looked into constitutive activation of transducin by wild-type and mutant salamander UV cone pigments. The apoproteins of the UV cone pigment and E113Q mutation (rhodopsin numbering) are constitutively active, that is, they can activate transducin in the absence of chromophore and consequently light-independent. This activity is quenched upon binding 11-cis retinal. We have also found a triple mutant of this UV cone pigment (F86L, T88P, S118T, using bovine rhodopsin numbers) that is both constitutively and dark active. This mutant is able to bind 11-cis retinal to form a pigment that absorbs primarily in the UV region (374 nm); however, unlike the wild-type, the Schiff base is able to be protonated when the pH is lowered producing a 475 nm absorbing species. This pigment is able to activate tranducin independent of light. Interestingly, the apoprotein of this triple mutant is also quite constitutively active. Generating a pigment of this triple mutant with the 9-desmethyl analog of 11-cis retinal completely abolishes dark activity and renders the pigment light sensitive similar to the wild-type UV pigment with 11-cis retinal. Also, the spectrum of the 9-desmethyl triple mutant UV pigment does not change between pH 5 and 7, again similar to the wild-type UV pigment.

P-108 A VISUAL PIGMENT EXPRESSED IN BOTH RODS AND CONES

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Rods and cones contain closely related but distinct G-protein-coupled receptors, opsins, which have diverged to meet the differing requirements of night and day vision. However, we have the first evidence for an exception to that rule. The retina of the aquatic phase salamander contains five types of photoreceptor cells: red and green rods, and red, blue and UV-sensitive cones but only four opsins. Results from immunohistochemistry, spectrophotometry and single cell RT-PCR demonstrate that the green rods and blue-sensitive cones contain the same opsin. This opsin displays a sequence and biochemical properties of a typical cone pigment. In contrast, the two cells express distinct G-protein transducin α-subunits, rod α-transducin in green rods and cone α-transducin in blue-sensitive cones. The different transducins do not appear to markedly affect photon sensitivity or response kinetics in the green rod and blue-sensitive cone. With a cone pigment but a rod transducin and rod morphology, the green rod may represent an intermediate in the cone to rod evolution. In land phase salamander, however, the density of green rods in the retina increases while the blue-sensitive cones disappear, suggesting that blue-sensitive cones and green rods may be responsible for blue-sensitive vision in different environments in this species.

P-109 COULD DIFFERENCES IN BLEACHING ADAPTATION IN RODS AND CONES ARISE FROM THEIR TRANSDUCINS?

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Capture of a photon by a visual pigment molecule activates a biochemical cascade that results in an electrical signal, thus initiating the visual process. Photoexcitation of the pigment also causes the retinal chromophore to dissociate from opsin. After exposure to very bright light, rods are desensitized to an extent far greater than expected by the decrease in quantal catch alone because opsin persistently activates the phototransduction cascade. Although the activity of a single opsin is weak, the total activity arising from over ten million molecules becomes significant. This behavior is known as "bleaching" adaptation. In salamander red-sensitive cones, β-ionone has been shown to relieve bleaching adaptation by quenching the activity of opsin. Apparently β-ionone changes the conformation of opsin by settling into its chromophoric binding pocket without the formation of a covalent bond. Surprisingly, β-ionone was observed to exacerbate bleaching adaptation in red rods. The contrasting behavior in the two cell types was attributed to a difference in the types of opsins that they contain. However, β-ionone could induce a similar conformation in both red-sensitive cone and red rod opsins that is capable of activating rod transducin but not cone transducin. To test this hypothesis, we are determining the effect of β-ionone on the sensitivity of partially bleached green rods and blue-sensitive cones in salamander, which contain the same pigment coupled to different transducins. Despite the presence of a cone-like opsin, β-ionone produced a loss in sensitivity in partially bleached green rods. This result suggests that transducin type may play a role in distinguishing bleaching adaptation in rods from that in cones.

P-110 CALCIUM-DEPENDENT ASSEMBLY OF CENTRIN-G-PROTEIN COMPLEX IN MAMMALIAN PHOTORECEPTORS

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Vertebrate photoreceptor cells are highly polarized neurons, which consist of morphological and functional distinct cellular compartments. The outer segment contains on specialized disc-like membranes the G-protein coupled transduction cascade, one of the best studied examples of a G-protein coupled system. In the inner segment, *de novo* biosynthesis of the components of the transduction machinery takes place. Any intracellular exchange between the inner and outer segments occurs through the slender non-motile connecting cilium. The molecular mechanisms underlying translocations between cellular compartments have been of general interest and the unidirectional outward transport of the visual pigment rhodopsin, for which motor proteins have been recently identified, has become a central paradigm in the field.

Here we deal with the visual G-protein transducin and its light-induced and bi-directional exchange through the cilium. We demonstrate that transducin forms a Ca^{2+}-dependent complex with the cytoskeletal protein centrin1. This Ca^{2+}-binding protein, a member of the parvalbumin superfamily, is activated by Ca^{2+}-induced conformation changes. Immunoelectron microscopy reveals now that transducin and centrin1 co-localize in a specific domain of the photoreceptor connecting cilium. We show by co-immunoprecipitation, overlay essays, size exclusion chromatography, and kinetic light scattering experiments that Ca^{2+}-activated centrin1 binds with high affinity and specificity to transducin. The assembly of the centrin-G-protein complex is mediated by the transducin βγ complex. The Ca^{2+}-dependent assembly of a G-protein with centrin is a novel aspect of the supply of signaling proteins in sensory cells, and a potential link between molecular translocations and signal transduction in general.

P-111 ARRESTIN AND ITS SPLICE VARIANT P44: MECHANISM AND BIOLOGICAL ROLE OF THEIR INTERACTION WITH RHODOPSIN

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Deactivation of G-protein coupled receptors relies on a timely blockade by arrestin. However, under dim light conditions, virtually all arrestin is in the rod inner segment and the splice variant p^{44} (Arr^{1-370A}) is the stop protein responsible for receptor deactivation. Using size exclusion chromatography and biophysical assays for membrane bound protein-protein interaction, membrane binding and G-protein activation, we have investigated the interactions of Arr^{1-370A} and proteolytically truncated Arr^{3-367} with rhodopsin. We find that these short arrestins do not only interact with the phosphorylated active receptor but also with inactive phosphorylated rhodopsin or opsin in membranes or solution. Due to the latter interaction they are not soluble (like arrestin) but membrane bound in the dark. Upon photoexcitation, Arr^{3-367} and Arr^{1-370A} interact with prephosphorylated rhodopsin faster than arrestin and start to quench G_t activation on a subsecond time scale. The data indicate that in the course of rhodopsin deactivation, Arr^{1-370A} is handed over from inactive to active phosphorylated rhodopsin. This mechanism could provide a new aspect of receptor shut off in the single photon operating range of the rod cell.

P-112 ACTIVATION AND DEACTIVATION OF RHODOPSIN BY LIGHT

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After light-induced 11-*cis*-/ all-*trans*-retinal isomerization and subsequent proton transfers, rhodopsin forms the active G-protein binding Meta II state. When Meta II is photolyzed by blue light, this pathway is apparently reverted and rhodopsin photoregenerated. However, the product formed (P_{500}) is different from the ground state [1] by the following observations: i) the ground state fingerprint of 11-*cis*-retinal is not present in the infrared spectra; ii) extraction of retinals from P_{500} predominantly yields all-*trans*-retinal; iii) P_{500} is similar to the thermal decay product Meta III. Both P_{500} and Meta III can be photoconverted to Meta II with the same FTIR difference spectrum and extraction of retinals from both products yields predominantly all-*trans*-retinal. The data indicate a "second switch" between active and inactive conformations, which operates without isomerization around the C_{11}-C_{12} double bond. This emphasizes the exclusivity of the ground state, which is only accessible by metabolic regeneration with 11-*cis*-retinal. Infrared difference spectroscopy and biochemical methods [2] also show that in rhodopsin bound with ring constrained 11-*cis*-retinal analogs the chromophore undergoes photoisomerization; however, it remains marginally active because the chromophore-induced conformational change of the opsin moiety does not occur. Modeling the locked chromophore analogs in the active site of rhodopsin suggests that the β-ionone ring rotates but is largely confined within the binding site. These results suggest that the native chromophore *cis-trans* isomerization is merely a mechanism for repositioning of the β-ionone ring that ultimately determines receptor activation.

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P-113 METARHODOPSIN – MEDIATED APOPTOSIS IN *DROSOPHILA* PHOTORECEPTORS

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The current challenge is to identify and characterize the molecular pathways and kinetics of different phases of metarhodopsin-mediated apoptotic retinal degeneration in *Drosophila*, as well as to understand how the visual signaling machinery regulates this process. Previous to [1] we have shown that the internalization of phosphorylated metarhodopsin/Arr2 complexes within one day after application of the trigger (blue light λmax = 440 nm) is a necessary step for apoptosis in the $rdgC^{306}$ mutant that lacks the rhodopsin-specific phosphatase. Here, we demonstrate that the commitment phase of apoptotic retinal degeneration in $rdgC^{306}$ lasts 2.3 days. During this phase the photo-conversion of metarhodopsin to rhodopsin by orange light (λmax = 600 nm) halts the degeneration. After 2.3 days the photoreceptors enter an irreversible execution phase, resulting in significant changes in the fly eye morphology. The commitment phase was found to be slow, but very reliable. Such reliability suggests a strong feedback control mechanism that provides long latency by a highly cooperative initiation switch. Phosphorylation of Arr2 may play a role in the execution of this mechanism since the commitment phase cannot be observed in an Arr2 mutant $arr2^{1}$ $rdgC^{306}$ that lacks the arrestin phosphorylation site.

To identify genetic elements of the apoptotic pathway, we monitored changes in transcript levels of 14,011 genes during the commitment phase of retinal degeneration in $rdgC^{306}$ by using the Affymetrix microarray technology. A cluster of genes including various exo and endopeptidases are down regulated in $rdgC^{306}$ (but not in wild-type flies) 2 days after photo-conversion of rhodopsin to metarhodopsin. These results establish a functional link between generation of phosphorylated metarhodopsin in $rdgC^{306}$ and transcription regulation of proteolytic enzymes that may play a role in mediating of neurodegenerative processes.

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P-114 ROLE OF A2E IN THE PATHOGENESIS OF AGE-RELATED MACULAR DEGENERATION

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Deposition of a fluorophoric material, known as lipofuscin, in retinal pigment epithelium (RPE) cells has been speculated to be one of the biomarkers of age-related macular degeneration. One of the fluorophores of lipofuscin has been characterized as A2E, a pyridinium bisretinoid.[1] Its cationic nature along with two hydrophobic retinal chains suggests that it can disrupt the membrane integrity by its detergent-like activity and can thus cause cellular damage. With this notion, we studied in detail the interaction between A2E and the model membranes of different lipid compositions using fluorescence steady-state and fluorescence anisotropy measurements. We showed that A2E induces vesicular to micellar transition upon incorporation into the lipid bilayer and membrane solubilization accompanies leakage of the vesicular contents.[2] We then focused on how A2E induces apoptosis in RPE cells. Human RPE cells (ARPE-19) were treated with different amounts of A2E (25, 75 and 100 μM; A2E contained all the epoxide forms). Though a decrease in cell viability (~60 % viability after 24 h with 100 μM A2E) was observed with the increase in A2E concentration, no fragmentation of DNA was observed in apoptotic DNA ladder experiment. Fluorescence microscopy also didn't show any DNA condensation. Treatment with 100 μM A2E released the cytochrome c into the cytosol, but loss of mitochondrial membrane potential was not observed. At that condition, outer membrane integrity of mitochondria was completely lost. Caspase-3 activity was not observed in the cell lysate. However processing of caspase-8 was found. Treatment with 100 μM A2E showed a 70 % increase in the reactive oxygen species (ROS) as compared with the control cells. Enzymes those are known to counteract ROS were also monitored in the cell lysate. These results suggest an involvement of mitochondria in A2E-induced apoptosis process.

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P-115 INCREASED ACTIVITY IN RHODOPSIN POINT MUTANTS ASSOCIATED WITH RETINITIS PIGMENTOSA

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Rhodopsin is the visual pigment of rod phototoreceptor cells in the vertebrate retina which mediates dim light vision. This protein is a seven transmembrane helical receptor belonging to the G-protein-coupled receptor (GPCR) superfamily, for which it is widely used as a structural and functional model. We have studied point mutations in rhodopsin associated with the retinal degenerative disease retinitis pigmentosa (RP) by using a combination of molecular biology and biophysical techniques, namely site-directed mutagenesis and spectroscopic techniques. This approach has allowed new insights into structure-function relationships in rhodopsin to be obtained. We have expressed and characterised mutations associated with RP in the different domains of the protein: transmembrane (M44T, G114D, P171Q, and V137M), intradiscal (G106W) and cytoplasmic (L328P, A346P).

The ability of the proteins to bind 11-*cis*-retinal was determined by means of UV-Vis spectroscopy, and the functionality of the mutant proteins was determined from its ability to activate the G protein transducin, as measured by fluorescence spectroscopy. The RP-related mutations G106W, G114D and P171Q cause misfolding of the mutant proteins, this resulting in impaired 11-*cis*-retinal binding and absence of chromophore regeneration. Rhodopsin mutants M44T, V137M and L328P regenerated in the presence of 11-*cis*-retinal at a similar level than the *wild type* protein, but the initial velocity of the G-protein activation process after photoactivation was increased. This hyperactivity may disturb the optimal balance among the different protein components of the visual phototransduction cascade, activating the apoptotic mechanism wich leads to photoreceptor cell death in RP disease.

P-116 PHOTOTROPINS: LIGHT-DRIVEN MOLECULAR SWITCHES IN PLANTS

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Phototropins (phot1 and phot2), the plant blue light receptors for phototropism, chloroplast movement, and stomatal opening, are flavoproteins that contain two 12kD FMN-binding domains, LOV1 and LOV2, at their N-terminus, and a serine-threonine protein kinase domain at their C-terminus. The LOV domains are members of the PAS protein superfamily that includes Photoactive Yellow Protein (PYP). Phototropins autophosphorylate, and it has been proposed that they function as light-activated serine kinases. *E-coli* expressed LOV domains bind FMN, are photochemically active, and absorb maximally at 450 nm. All LOV domains undergo cyclic photoreactions. In phot1-LOV2 a triplet state (650nm, <30 ns), decays in 4 us into a 390 nm intermediate shown to be a protein-chromophore cysteinyl adduct (cys39) at the C(4a) of FMN that returns to the ground state (~ 30s). The light-minus-dark FTIR difference spectra of phot1-LOV2 shows the disappearance of bands at 1580, 1550, and 1350 cm^{-1} that originate from, or are strongly coupled to, the N5=C(4a) stretching vibrations, consistent with the perturbations expected upon C(4a) adduct formation. Assignment of these negative difference FTIR bands to native chromophore vibrations is based on the alignment pre-resonance Raman bands of LOV2. Prominent positive bands include a doublet at 1516 and 1536 cm^{-1} and one at 1375 and 1298 cm^{-1}. Normal-mode vibrational-frequency calculations for normal and isotopically substituted lumiflavin and lumiflavin with a sulfur attached at the C(4a) position agree with many of the positive and negative bands observed in the difference spectra and are consistent with the assignment of the long-lived cysteinyl adduct. Circular dichroism data suggest that protein α-helicity is lost upon light-irradiation. CD of both protein and chromophore follow identical relaxation kinetics suggesting a common rate-limiting step in the back-reaction The relaxation rate in D_2O is three-fold slower than in H_2O, indicating involvement of intramolecular proton transfers.

P-117 KINETICS OF PROTON UPTAKE AND RELEASE BY THE PHOTORECEPTOR PYP

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The kinetics of the photocycle and of proton uptake and release in the photoreceptor Photoactive Yellow Protein (PYP) from *Ectothiorhodospira halophila* were measured by time-resolved absorption spectroscopy. The proton kinetics were monitored in the external medium at different pH values using the dyes bromocresol purple, cresol red and alizarin red. Protonation of the p-hydroxycinnamoyl chromophore was monitored by the formation of the blue-shifted I_2 intermediate. For wild-type, the kinetics of I_2 formation correlates well with that of the proton uptake signal (500 µs at pH 6.2). For the mutant E46Q, in which the putative internal donor E46 is absent, the formation of I_2 is accelerated, as is the proton uptake from the external medium (110 µs at pH 6.3). For the mutant E46A the kinetics are even faster but remain correlated (spectral changes and pH changes) (20 µs at pH 8.3). The kinetics of proton release is in all cases strictly correlated with the multiphasic reformation of the initial PYP ground state from I_2. These results suggest an alternative model for the chromophore protonation: either by the internal proton donor E46, or via protons from the aqueous medium, as a consequence of the protein conformational change, rather than as a trigger for that change.

P-118 PROTONATION CHANGES AND DYE BINDING BY PHOTOACTIVE YELLOW PROTEIN IN WILD-TYPE AND IN E46 MUTANTS

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We studied the kinetics of proton uptake and release by Photoactive Yellow Protein (PYP) from *Ectothiorhodospira halophila* in wild-type and the E46Q and E46A mutants by transient absorption spectroscopy with the pH-indicator dyes bromocresol purple or cresol red in unbuffered solution. In parallel, we investigated the kinetics of chromophore protonation as monitored by the rise and decay of the blue-shifted state I_2 (λ_{max} = 355 nm). For wild-type the proton uptake kinetics is synchronized with the fast phase of I_2 formation (τ = 500 µs at pH 6.2). The transient absorption signal from the dye also contains a slower component which is not due to dye deprotonation, but is caused by dye binding to a hydrophobic patch that is transiently exposed in the structurally changed and partially unfolded I_2 intermediate. SVD analysis moreover indicates the presence of two components in the dye signal: protonation and dye binding. The dye binding has a rise time of about 4 ms and is coupled kinetically with a transition between two I_2 intermediates. In the mutant E46Q, which lacks the putative internal proton donor E46, the formation of I_2 is accelerated, but the proton uptake kinetics remains kinetically coupled to the fast phase of I_2 formation (τ = 100 µs at pH 6.3). For this mutant the protein conformational change, as monitored by the dye binding, occurs with about the same time-constant as in wild-type, but with reduced amplitude. In the alkaline form of the mutant E46A the formation of the I_2-like intermediate is even faster as is the proton uptake (τ = 20 µs at pH 8.3). No dye binding occurred in E46A suggesting the absence of a conformational change. In all the systems proton release is synchronized with the decay of I_2. Our results support mechanisms in which the chromophore of PYP is protonated directly from the external medium rather than by the internal donor E46.

P-119 MIMIC OF THE PHOTOCYCLE OF PHOTOACTIVE YELLOW PROTEIN BY pH JUMP PROTEIN FOLDING EXPERIMENTS

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The blue light receptor photoactive yellow protein (PYP) displays rhodopsin-like photochemistry based on the *trans* to *cis* photoisomerization of its *p*-coumaric acid chromophore. Here, we report that protein refolding from the acid denatured state of PYP mimics the last photocycle transition in PYP. This reveals a direct link between transient protein unfolding and photosensory signal transduction. We utilize this link to study general issues in protein folding. Chromophore *trans* to *cis* photoisomerization in the acid denatured state strongly decelerates refolding, and converts the pH dependence of the barrier for refolding from linear to non-linear. We propose transition state movement to explain this phenomenon. The *cis* chromophore significantly stabilizes the acid denatured state, but acidification of PYP results in the accumulation of the acid denatured state containing a *trans* chromophore. This provides a clear example of kinetic control in a protein unfolding reaction. These results demonstrate the power of PYP as a light-triggered model system to study protein folding.

P-120 CRYSTAL STRUCTURE OF R52Q MUTANT OF PHOTOACTIVE YELLOW PROTEIN

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Photoactive yellow protein (PYP), involved in the negative phototaxis of *Ectothiorhodospira halophila*, is a water-soluble photoreceptor protein. PYP has an α/β-fold structure composed of 125 amino acids and *p*-coumaric acid as a chromophore. Although the phenolic oxygen ($O_{4'}$) of the chromophore is deprotonated in the dark, it is protonated at M intermediate during the photocycle. Moreover, during this cycle, guanidino group of R52, which locates near the chromophore, is exposed to the solvent (*1*). To investigate the role of R52, R52Q mutant was prepared and subjected to spectroscopic measurements. Although the absorption maximum of R52Q (447 nm) is almost identical to that of wild type (446 nm), p*K*a of the chromophore is increased from 2.9 to 4.0 by the mutation. In order to interpret such alteration on the basis of the molecular structure, we analyzed the crystal structure of R52Q mutant. In wild type, guanidino group of R52 forms hydrogen bonds with amide oxygens of T50 and Y98. The distances from $N_{\eta 1}$ of R52 to O of T50 and from $N_{\eta 2}$ of R52 to O of Y98 are 2.9, 2.7 Å, respectively. However, these hydrogen bonds are broken by the mutation. The distances from $N_{\varepsilon 2}$ of Q52 to O of T50 and to O of Y98 are extended to 4.3 and 6.8 Å, respectively, and one water molecule coordinates in this space. This water molecule forms hydrogen bonds with $N_{\varepsilon 2}$ of Q52 and O of V66; both of the distances are 3.1 Å. These results indicate that the increase of p*K*a of the chromophore caused by the mutation to R52 is brought about by the breakage of the hydrogen bonds. Therefore, we assume that R52 regulates the proton transfer from E46 to the chromophore in the photocycle by changing its conformation.

(1)Genick *et al.* (1997) *Science 275*, 1471-1475

P-121 DIRECT OBSERVATION OF LIGHT-INDUCED GLOBAL CONFORMATIONAL CHANGE OF PHOTOACTIVE YELLOW PROTEIN

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On photon absorption, photoactive yellow protein (PYP) undergoes the photoreaction cycle. The last intermediate (PYP_M), whose lifetime is ~100 ms, is considered to be the key intermediate for the physiological function of PYP. On formation of PYP_M, a proton at Glu46 is transferred to the chromophore, and the hydrogen-bonding network around the phenolic oxygen of the chromophore is largely altered. As a result, the global conformational change of PYP molecule takes place. The light-induced global conformational change of PYP was directly detected by small-angle X-ray scattering experiments (SAXS).

PYP_M was stabilized by acidification or enzymatic truncation of N-terminal 6, 15 or 23 amino acid residues (T6, T15, and T23, respectively) and accumulated under the continuous illumination for SAXS measurements. The radius of gyration (R_g) was estimated by Guinier plot, and the globularity of the molecule was examined by Kratky plot. The illumination of T6, T15, and T23 caused the increase of R_g by 1.1, 0.7, and 0.7 Å, respectively, suggesting that PYP swells upon formation of PYP_M. Because T23 lacks most part of N-terminal loop, the structural change of main part is responsible for the R_g increase by 0.7 Å, and that of N-terminal 15 amino acid residues for the additional R_g increase by 0.4 Å. The band widths of R_g-normalized Kratky plots for PYP_M and $T6_M$ were larger than their dark states, while those of $T15_M$ and $T23_M$ were comparable to their dark states. Therefore, structural change of N-terminal 15 amino acid residues alters the shape of PYP. These findings suggests that N-terminal region moves outward upon formation of PYP_M.

P-122 SELF-ASSOCIATION OF BOVINE VISUAL ARRESTIN AS REVEALED BY SMALL ANGLE X-RAY SCATTERING

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Light-activated rhodopsin is phosphorylated and binds to arrestin. The oligomeric states of bovine visual arrestin in solution were studied by small-angle X-ray scattering. The Guinier plot of arrestin at low concentration (< 0.5mg/ml) can be approximated with a straight line up to $Q^2 = 0.01$. At high concentration, however, there is an inflection in the Guinier plot, and the inflection shifts toward low Q side as the concentration increases. These facts indicate that arrestin in solution shows concentration-dependent self-association. The concentration-normalized intensity at zero angle, I(0)/conc, is a measure of molecular weight of the scatterer. Using ovalbumin as a molecular weight standard, it was found that arrestin varied from monomer to tetramer depending on the concentration. I(0)/conc decreased at high salt concentration, but was independent of the temperature, suggesting that the self-association of arrestin is mainly due to electrostatic interaction but not hydrophobic interaction. The simulation analysis of the concentration-dependent increase of I(0)/conc demonstrated that the tetramerization is highly cooperative, and that the concentration of arrestin monomer remains at an almost constant level even if the total concentration of arrestin fluctuates within the physiological range. A binding assay of phosphorylated rhodopsin and arrestin indicated that phosphorylated rhodopsin binds to arrestin in the monomer form. Taken together, these results suggest that arrestin monomer is reserved in the tetramer to cope with a rapid increase of phosphorylated metarhodopsin II.

P-123 PHOTOACTIVE YELLOW PROTEIN ANALOGUES WITH HALOGENATED CHROMOPHORES

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Photoactive Yellow Protein (PYP) is a blue-light photoreceptor, which has a *p*-coumaric acid (H-*p*CA) as a chromophore, covalently bound to Cys69 via a thiol ester. In PYP_{Dark}, the phenolic hydroxyl group of the chromophore is deprotonated and forms a hydrogen-bonding network with amino acid residues. Free H-*p*CA in water at neutral pH absorbs maximally at 286nm; however, within the chromophore-binding pocket in the apoprotein, the absorption of the chromophore is red-shifted to 446 nm. Previous studies about this large red shift of chromopore in PYP paid attention to disrupting and altering the hydrogen-bonding network. This study is focused on the role of the π-electron system of the chromophore in color tuning.

In this study, we tried to design and prepare halogenated chromophores whose α-proton of the carboxylic group was substituted with a halogen atom (F or Br). Two main effects of substitution, electronic and steric, are expected. Those were caused by the electronegative and van der Waals interactions.

We studied the spectroscopic properties of the analogue chromophores and the reconstituted PYPs. At basic conditions, free F- *p*CA was 13nm blue-shifted compared to that of H-*p*CA, while the λ_{max} of Br- *p*CA was quite similar to that of H-*p*CA. However each λ_{max} of the both F- and Br-analogues in the protein, PYP, appeared at same position, 446nm, which was the same as that of native PYP, showing that red-shift of F-*p*CA is larger than those of Br- and H-*p*CA. Moreover, the pK values of both F- and Br-*p*CA in base titration hardly distinguished from H- *p*CA's.

These results suggest that the larger red-shift of F-*p*CA is due to change of π-electron systems by the highelectronegativity of F atom, not due to the streric effect, because all pK values of free chromophores are similar to each other. This might indicate that other specific interactions between the substituent and protein moiety cause another 13 nm red-shift of λ_{max} in F-PYP.

P-124 SOLUTION STRUCTURE AND DYNAMICS OF PHOTOACTIVE YELLOW PROTEIN AS STUDIED BY MOLECULAR DYNAMICS SIMULATION

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To elucidate solution structure and dynamics of photoactive yellow protein (PYP) and two photocycle intermediates L and M, we carried out molecular dynamics simulations. The initial structure of each state was taken from the corresponding X-ray structure. The protein was solvated in a droplet water with a radius of 30 angstrom. From comparison of 10 ns simulations for the three states, it was found that they have significantly different dynamical properties, which were examined mainly from the data of root-mean-square fluctuation (RMSF), and variance-covariance analysis followed by the principal component analysis. These data indicate that the structural changes (photoisomerization and protonation) of the chromophore cause significant modifications in the dynamics of the whole protein. In general, low frequency vibrational modes of a protein is expected to be responsible for the occurrence of its function. The results for the principal component analysis revealed that the M state, a putative signaling state, has a characteristic fluctuation mode which corresponds to the motion bisecting the whole protein at the boundary of the chromophore binding pocket. The presence of such a unique mode may contribte to binding with transducin.

A NOVEL RETINAL PROTEIN, *SEATTLEOPSIN*

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----NAMTINABDLAEVZSLTABLNCZYLRIKEALEIEVRSANAALVAREZANAANDRESHLLYA
TKINSNSERGEIBALASHVNITINBALIGAERNSTBAMBERGFRANZBARTLDNALDBASHFRDR
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NKRZYSZTFBRYLSTEPHENBDYGERGBELDTVLKERBSSRSEANNCATTLICPCHENANGELAC
RCELLICARTERCRNWALLRSALIECRCHMICHAELCSANVICHSMADEWILLEMDEGRIPNRBE
RTDENCHERANDRASDERANDREIDIMAEVMARTADMINGEZSEANEANNADRZHKTHMASEBRE
YMARTINENGELHARDLIVERERNSTLASZLFABIANMARCFACCITTIDAVIDFARRENSEFFR
YFASICKSLAWMIRFILIPEKNATHANFISHKINHENRYFNGKENNETHFSTERLAFFRITZEYK
ARIFIMTNAKFIKAYIFRTANIFLRIANGARCZAREKKLASGERWERTRBERTGLAESERRBERT
GRIFFINARMILAGIARRREZNICKVAMARILYNGNNERMENACHEMGTMANPALHARGRAVESH
IGEHIKHAYASHIACHIMHEBERLEMARTINHECKKLAASHELLINGWERFRLFHERRMANNDIT
HHERZFELDAYHILLEBRECHTTAKAHIRHIRANSAMHISATMIWTERHFFKLASPETERHFMAN
NHARVEYIANMINHLIAHFENDAVIDHNTAMESHRLEYYASSHIIMAMTYSHIKAZIMANISHIE
LENRAIMASHEVAMASAIISHIGRMASAYKIIWAMTRASMSENSENTSHIAKIKAKITANIYANN
ISKALAIDZIDISNAKIKAMHIDEKIKANDRIELENAKARNAKHVAMIKIKATAKABRADLEYKE
LEMENLASKESZTHELYIANDREYKHDNVGBINDKHRANAALEANDERKISELEVDAVIDKLIGE
RMASAHIRKNELENAKRCHEMSKAYATSTMKYAMAMITSMASAKYANAGISNAKRANEMARKKRE
BSVLADIMIRKKSAMASATKMACHIEHDLANDAANSLANYIDANNYLETRNEAAMESLEWISYAN
LIANGPALLIEBMANMIALHARTMTLECKEHANLGTENBRGEVGENIYLKASHEVIANINGMAAK
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DMATHIESDAVIDMAZERALLSHAMCBEEHNMCINTYREESTHERNACHLIELVNAGARAANHIR
SHINAKAGAWAMASASHINAKAGAWAKINAKANISHIMARIANAWRTALEEYNFRIEVDANIELP
RIANPALRMSHARALDTTESTEVEPADRSKRZYSZTFPALCZEWSKIDHAIPANRANGAPARTHA
ALEPERALVAREZTHMASPEHLMANNALEANDERPLVERMLLERLAVISIERRAMSDANIELRIC
HTERKEVINRIDGEEGLFRITTERPHYLLISRBINSNSHAHABRHANIMANSHADISANFRDRHM
ANHNSAARITHMASSAKMARMINRSAKRAIDAVIDSAMMETHNSASAKINAMISAYANSANDRAS
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ECKENISEFFSTARTSRIRAMSBRAMANIAMYKISDMINRSGIHARAACKSLLIVANMASATSMI
ISTVANSZNDIEMADTAKHRSHIDYSKETAKAHASHIRIETANAKADAVIDTELLERAKIHISAT
ERAKITAANDREWTERENTISERGTITTRMTYKITSDAGERGETRNERGYRGYVARBELENVAZA
RAEANVELPPILLAIREINERVGELWEIWWANGYAIEWANGLIVERWEINGARTKEVINWISEQI
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ESHZYKYAMAKAZYSHIHARALIZHLASZLZIMANYI---

Can you find your motif?

AUTHOR INDEX